

MODULATION OF POLYMORPHONUCLEAR NEUTROPHIL RESPONSE  
TO N-FORMYL-L-METHIONYL-L-LEUCYL-L-PHENYLALANINE

1988

LINNEKIN



Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE <b>NOV 1988</b>		2. REPORT TYPE <b>N/A</b>		3. DATES COVERED <b>-</b>	
4. TITLE AND SUBTITLE <b>Modulation of Polymorphonuclear Neutrophil Response to N-formyl-1-methionyl-1-leucyl-1-phenylalanine</b>				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) <b>Uniformed Services University Of The Health Sciences Bethesda, MD 20814</b>				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT <b>Approved for public release, distribution unlimited</b>					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT <b>SAR</b>	18. NUMBER OF PAGES <b>214</b>	19a. NAME OF RESPONSIBLE PERSON
a. REPORT <b>unclassified</b>	b. ABSTRACT <b>unclassified</b>	c. THIS PAGE <b>unclassified</b>			



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Title of Thesis: Modulation of Polymorphonuclear Neutrophil Response  
to N-formyl-1-methionyl-1-leucyl-1-phenylalanine

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## ABSTRACT

Title of Dissertation:

Modulation of Polymorphonuclear Neutrophil  
Responses to N-formyl-l-methionyl-l-leucyl-l-  
phenylalanine

Diana Linnekin, Doctor of Philosophy, 1988

Dissertation directed by:

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N-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) is a potent stimulant of migration and superoxide production by polymorphonuclear neutrophils (PMNs) from many species. Dog and rhesus monkey PMNs are poorly responsive to FMLP and believed not to express the formylpeptide receptor. The objectives of these studies were to determine if PMNs from dogs and rhesus monkeys could be modified with certain agents to migrate or generate an oxidative burst in response to FMLP.

Pretreatment of dog PMNs in vitro with 1 nM PMA, a protein kinase C (PKC) activator, produced a 91.7% increase in migration to 1 pM FMLP ( $P < .005$ ). In vitro pretreatment with A23187 did not alter migration of dog PMNs to FMLP. Both PMA and A23187 induce expression of intracellular formylpeptide receptor stores in human PMNs. These data suggest PMA induced enhancement in migration to FMLP is not mediated through increases in formylpeptide receptor numbers but through the effects of PKC on receptors on the dog PMN membrane. Subsequent studies established that dog PMNs migrated and generated an oxidative burst to high concentrations of FMLP, to analogs with high

affinity for the formylpeptide receptor and to E. coli filtrates. These responses were inhibited over 50% by pertussis toxin and 43.8% by a formylpeptide receptor antagonist, demonstrating the existence of small numbers of formylpeptide receptors as potential PKC targets.

The effects of in vitro and in vivo treatment with granulocyte-macrophage colony stimulating factor (GM-CSF) on rhesus monkey PMNs were assessed. In vitro exposure of rhesus monkey PMNs to rhGM-CSF (10 U/ml) led to a 93% increase in migration to FMLP after 5 minutes pretreatment while a 120 minute pretreatment increased oxidative responses by 247%. Subcutaneous administration of rhGM-CSF in vivo led to the production and release of a more aggressive population of PMNs from the bone marrow which had a 179% increase in migration to FMLP.

These studies demonstrate that species poorly responsive to FMLP can be induced to a responsive state after pretreatment with appropriate priming agents both in vitro and in vivo and suggests these events may occur and be of significant benefit to the host during inflammation.



Modulation of Polymorphonuclear Neutrophil Responses  
to N-formyl-l-methionyl-l-leucyl-l-phenylalanine

by

Diana Linnekin

Dissertation submitted to the Faculty  
of the Department of Physiology  
Graduate Program of the Uniformed Services University  
of the Health Sciences in partial fulfillment of the  
requirements for the degree of  
Doctor of Philosophy, 1988

## ACKNOWLEDGEMENTS

The multitudes of people who gave me the benefit of their experience, support and kindness these past four years have my most sincere gratitude. Dr. Gene Murano, my major advisor, has been a wonderful research advisor. He always found time to review data and offer helpful insights as well as encouragement. Thank you to Dr. Tom MacVittie for suggesting the graduate program at USUHS to me and for offering a tremendous amount of personal and scientific support, as well as for access to the resources at AFRRI. The committee chairperson, Dr. Jim Terris, has been incredibly helpful through every important milestone in the graduate program and I could never thank him enough for his support and advice at each of those points. Doctors Tom Cote and Greg Mueller, were both committee members and each made many helpful suggestions about the design and execution of my project. Thank you to Dr. Cote for the pertussis toxin used in the studies. I have a tremendous amount of respect and admiration for the scientific expertise of each of the members of my committee. I thank each of you very much for what you have taught me about research and academics.

I would like to thank Dr. Charles Bowles, whom I worked for prior to starting graduate school. Dr. Bowles spent many hours reviewing data, manuscripts, the dissertation, offering helpful advice and provided endless amounts of encouragement.

Thank you to everyone in the Experimental Hematology at AFRRI for support these past four years. Specifically, I would like to thank Richard Brandenburg for procurement of samples of peripheral blood from dogs and for his infectious cheerfulness; to Gloria Contreras for putting up with my administrative deficiencies in such a nice way; John Darden for his expertise in operation of the EPICS V flow cytometer; Dr. Thomas Elliot for providing E. coli cultures absolutely crucial to the project and to Dr. Rod Monroy for providing samples from rhesus monkeys, for many hours reviewing manuscripts and for personal support. Additionally, I would like to extend my thanks to Dr. William Blakely for expertise in operation of the HPLC; Dr. Eric Holwitt for teaching me thin layer chromatography; Dr. Elaine Gallin for helpful suggestions; Dr. Dan Rotrosen for teaching me receptor binding assays; Dr. Linda Werlin for her time and patience while teaching me about the 'Ligand' program and to Dr. Liana Harvath who kindly offered me the use of her Optimax Image Analyzer as well as many hours of her time.

Lastly, I would like to thank my mother, father, brother and William Byrne for their love, patience, support and understanding. Reaching a milestone such as this is particularly joyful when shared with such wonderful people.



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## LIST OF ABBREVIATIONS

AFRRI	Armed Forces Radiobiology Research Institute
C5a	The cleavage product of the fifth component of complement
cGMP	Cyclic guanosine monophosphate
DAG	Diacylglycerol
dbc AMP	Dibutyryl cyclic adenosine monophosphate
DCF	2'7' Dichlorofluorescein diacetate
DMSO	Dimethyl sulfoxide
FCS	Fetal calf serum
FMLP	N-formyl-l-methionyl-l-leucyl-l-phenylalanine
FMLPB	N-formyl-l-methionyl-l-leucyl-l-phenylalanine benzylamide
FMLPP	N-formyl-l-methionyl-l-leucyl-l-phenylalanine-l-phenylalanine
FNLLP	N-formyl-l-norleucyl-l-leucyl-l-phenylalanine
FNLLPNLTL	N-formyl-l-norleucyl-l-leucyl-l-phenylalanine-l-norleucyl-l-tyrosyl-l-lysine
GM-CSF	Granulocyte-macrophage colony stimulating factor
GTP	Guanosine triphosphate
HBSS w/o	Hanks balanced salt solution without $\text{Ca}^{++}$ and $\text{Mg}^{++}$
HPLC	High pressure liquid chromatography
$\text{IP}_3$	Inositol tris (1,4,5)-phosphate
LIF	Leucocyte inhibitory factor
LPS	Lipopolysaccharide
$\text{LTB}_4$	Leukotriene $\text{B}_4$
MNCs	Mononuclear cells
MPO	Myeloperoxidase
NBT	Nitroblue tetrazolium
NDS	Normal dog sera
PAF	Platelet activating factor
$\text{PIP}_2$	Phosphatidylinositol 4,5-bisphosphate
$\text{PLA}_2$	Phospholipase $\text{A}_2$
PKC	Protein kinase C
PLC	Phospholipase C
PMA	Phorbol myristate acetate
PMNs	Polymorphonuclear neutrophilic leucocyte
PT	Pertussis toxin
RBCs	Red blood cells
RhGM-CSF	Recombinant human granulocyte-macrophage colony stimulating factor
SEM	Standard error of the mean
SOD	Superoxide dismutase
TLC	Thin layer chromatography
t-BOC-MLP	Tert-butyloxycarbonyl-l-methionyl-l-leucyl-l-phenylalanine
ZAS	Zymosan activated sera

## I. INTRODUCTION

### A. GENERAL BACKGROUND

Polymorphonuclear neutrophilic leucocytes (PMNs) are one of the first lines of defense against bacterial challenge. Consequently PMNs have developed a series of specialized functions designed to eliminate pathogenic organisms. These include adherence to endothelium, random and directed migration, receptor mediated binding of opsonized particles, phagocytosis, generation of reactive oxygen intermediates and release of hydrolytic enzymes. Stimulants of PMN function are both soluble and particulate. In vivo soluble mediators are from both intrinsic and extrinsic sources. Intrinsic mediators are those generated by the host and include C5a, leukotriene B<sub>4</sub> (LTB<sub>4</sub>) and platelet activating factor (PAF). Extrinsic mediators are those released by bacteria. A notable example is components of bacteria cell walls which are highly potent stimulators of inflammatory cell function. PMNs respond to gradients of chemoattractant by asymmetric orientation and locomotion from low to high concentrations of the stimulant. The capacity for directed migration through these concentration gradients is central to nonspecific immunity. The bactericidal potential of the PMN is of little value if it can not migrate to the foci of bacterial infection. PMNs bind, ingest and subsequently kill microorganisms. Bactericidal mechanisms include fusion of the azurophilic granules with the phagosome and the generation of an oxidative burst. Particulate stimuli such as opsonized particles, or soluble stimuli in the form of various chemotactic molecules stimulate a membrane bound oxidase which is NADP dependent. This oxidase reduces molecular oxygen

to a series of reactive oxygen intermediates, examples of which are superoxide anion, hydrogen peroxide and singlet oxygen. These compounds are highly unstable, therefore very reactive and serve as potent antimicrobial agents. The PMN cytoplasm contains catalase and other antioxidative enzymes designed to protect the inflammatory cell from these reactive compounds.

## B. EVENTS OF PMN ACTIVATION

### 1. PMN Chemotactic Receptors

In the mid 1970's Schiffmann et al. (1) described a family of formylated peptides having potent chemoattractant properties and proposed that these molecules mediate inflammatory cell stimulation by filtrates from E. coli cultures. Showell et al. (2) later reported these compounds as stimulators of degranulation and identified N-formyl-l-methionyl-l-leucyl-l-phenylalanine (FMLP) as a potent derivative. The significance of these findings was multifold. This gave researchers a powerful tool with which to assay parameters of PMN function such as motility, degranulation and generation of the respiratory burst. These peptides were also useful in identification of the structure activity relations for responses to formylated peptide derivatives.

In 1975 Williams et al. (3) reported the presence of receptors for formylated peptides on human PMN membranes and found that binding correlated with chemotactic response. These receptors, estimated at approximately 2000 per cell, reached equilibrium in 12 minutes at 37° C, had a Kd of 12 nM, were saturable, had the same structure activity



relation for binding as chemotaxis and were not present on purified lymphocytes or red blood cells. Koo et al. (4) suggested that human PMN membranes bound FMLP in a manner indicative of either negative cooperativity or receptor populations with 2 different affinities. Reports from other laboratories have confirmed the FMLP binding sites on PMNs of humans (3, 5, 6) rabbits (7) and guinea pigs (8). Current estimates of receptor numbers per cell range from the 2000 originally reported to 61,000. Estimated  $K_d$  values in whole cell preparations are variable and range from 0.077 nM to 200 nM. One explanation for the variation in binding results between laboratories may relate to differences in study protocols such as incubation buffers, temperature and means of separating bound from free ligand. Another explanation may relate to the observations of Zimmerli et al. (8) who demonstrated that exudation of PMNs increases receptor numbers for FMLP in guinea pigs and in humans. These data have important implications when comparing PMNs from the peritoneum of rabbits and guinea pigs, a common means of collection in these species, and those obtained from peripheral blood, a more common means of collection in humans. A third explanation for the variability in binding data may relate to differences in FMLP responsiveness between species (3, 7, 9, 10-17).

Receptors for other chemotactic factors such as PAF (18, 19), leukotriene  $B_4$  (20) and C5a (21) on PMNs from a variety of species have been identified.

## 2. PMN Signal Transduction and Second Messengers

The precise events involved in the stimulus response coupling of



chemotaxis, granule exocytosis and production of an oxidative burst have yet to be elucidated. Data generated in the past decade indicate that PMN responses are receptor mediated (3, 4, 7, 18-21), that chemotactic factor signal transduction appears to occur via a G regulatory protein (22-27) which is a pertussis toxin (PT) and cholera toxin substrate (28, 29), that phospholipase C is the effector molecule (30-34) and inositol 1,4,5 trisphosphate ( $IP_3$ ) (35) and diacylglycerol (DAG) (36) are second messengers. Recent articles have comprehensively reviewed these events in the PMN (37, 38). A brief summary of the present model is as follows. Occupancy of chemotactic receptors such as those for FMLP, PAF,  $LTB_4$  and others, causes binding of guanosine triphosphate (GTP) to a G regulatory protein which is apparently unique in the PMN. This G protein couples the receptor to a phospholipase C effector molecule. Phospholipase C (PLC) liberates  $IP_3$  and DAG from membrane stores of phosphatidylinositol 4, 5-bisphosphate ( $PIP_2$ ).  $IP_3$  is thought to raise intracellular calcium levels by stimulating calcium release from endoplasmic reticulum stores. An increase in intracellular calcium is one of the earliest events in receptor mediated PMN activation (39, 40). Degranulation and superoxide generation are dependent upon extracellular calcium (41) as are optimum chemotactic responses (42). DAG is the in vivo activator of protein kinase C (PKC) (36). Upon activation by DAG, PKC translocates from the cytosol to the membrane. In the membrane, PKC is cleaved from a precursor molecule having a molecular weight of 85 Kd to a 65 Kd form and is released into the cytoplasm (43). Cytoskeletal and membrane proteins are phosphorylated by PKC (44-47), however the physiological significance of the PKC substrates reported has yet to be fully defined. One major substrate thought to have an important role is a 38

Kd protein which Majerus et al. have identified as inositol (1, 4, 5) trisphosphate 5-phosphomonoesterase (30). Phosphorylation of this phosphatase by PKC has been shown to enhance degradation of  $IP_3$  to the less active  $IP_2$ . This may be the mechanism by which pretreatment of PMNs, platelets and other cell types with high doses of the PKC activator phorbol myristate acetate (PMA) may inhibit calcium mobilization, superoxide production or enzyme release (48-52).

Additionally, it has been shown that increases in intracellular calcium activates phospholipase  $A_2$  ( $PLA_2$ ) (53, 54). This phosphodiesterase liberates arachidonate from membrane phospholipid stores. Arachidonate is then metabolized to molecules of the prostanoid series by cyclooxygenase and to molecules of the leukotriene and eicosatetranoic acid series by lipoxygenase. Although the activation of  $PLA_2$  occurs unquestionably when cells are stimulated with calcium ionophores such as A23187 and ionomycin, Haines et al. (55) suggest that physiological stimulants such as FMLP, C5a, and others may not raise intracellular calcium sufficiently to activate  $PLA_2$  and elicit release of arachidonic acid. This paradox remains unresolved, but it is attractive to postulate that circumstances exist where PMNs release potent mediators such as  $LTB_4$  or eicosatetranoic acid derivatives and that these compounds serve as a means to autoregulate the inflammatory response.

### 3. Divergent Paths in PMN Signal Transduction

An interesting dilemma facing those studying signal transduction in the PMN is the means by which a stimulant, eg. FMLP, triggers the myriad of responses involved in inflammatory cell function and how this



relates to elicitation of the appropriate sequence of events. PMN function first involves migration to the site of host challenge, then bactericidal actions such as release of hydrolytic enzymes and production of reactive oxygen intermediates. The events regulating stimulation of motility versus degranulation and the oxidative burst are not well understood. As initially observed by Showell et al. (2), the  $ED_{50}$  for chemotaxis to FMLP is less than that for degranulation. Two hypothesis have been proposed to explain these findings. One theory proposes the existence of distinct receptor populations of 2 different affinities. The other possibility is the interconversion of one receptor between high and low affinity.

Perez et al. (56) demonstrated that a derivative of wheat germ agglutinin inhibited chemotaxis to FMLP but had no effect on migration to C5a. Additionally, degranulation in response to FMLP was not altered. These investigators subsequently established that wheat germ agglutinin derivative (WGA-D) bound on or near high affinity formylpeptide receptors and prevented receptor reexpression after internalization (57). These data are consistent with the interpretation that rapid responses such as degranulation involve engagement of low affinity receptors which are not actively recycled, while chemotaxis utilizes high affinity receptors and is a long term response necessitating receptor recycling to insure adequate receptor numbers to maintain motility. For example, monesin, a carboxylic ionophore for monovalent ions, was found to inhibit receptor mediated endocytosis and chemotaxis while shifting the dose response curve for the oxidative burst to FMLP only slightly to the right (58). Monesin appears to inhibit receptor processing in the Golgi. These data also imply, that when compared to the oxidative burst, chemotactic responses are more dependent on recycling of the putative high affinity receptor population.

Guanine nucleotides have been shown to convert high affinity binding to low affinity binding (4, 59). Sklar et al. (60) argue that all receptors are coupled to chemotaxis and the oxidative burst responses and divergent functional responses result from occupation of different numbers of receptors. Fast and slow dissociation components of receptor ligand interaction have been demonstrated as well as time dependent loss of the fast dissociating component. The slow dissociating form is proposed to be the high affinity receptor which is not regulated by guanine nucleotides and thus uncoupled from the receptor. This form is associated with the cytoskeleton and presumably internalized.

Treatment of rabbit PMNs with short chain aliphatic alcohols (2.5% n-propanol or n-butanol) was found to increase specific binding of FMLP by 2 to 3 fold (61). These observations were followed by studies conducted by Yuli et al. (62), demonstrating dose related changes in responses to FMLP which included increases in specific binding, an increase in chemotaxis and an inhibition of superoxide production. Based on these results it was suggested that high affinity receptors are linked to chemotaxis, low affinity receptors are linked to superoxide production, and that high and low affinity receptors are interconvertible. Affinity changes were proposed to result from alterations in membrane fluidity after treatment with the short chain aliphatic alcohols. Changes in membrane phospholipid metabolism do accompany cellular activation (63-66) which could increase membrane fluidity. Therefore, it is possible that high and low affinity receptors could be interconverted physiologically.



## C. MODULATION OF PMN FUNCTIONS

### 1. Receptor Modulation

As discussed, PMN stimulus-response coupling involves engagement of receptors for specific mediators, production of appropriate second messengers and translation via different effectors into a myriad of responses. Regulation of chemotaxis is particularly complex given that the directional component of this response is controlled by the concentration gradient of the stimulant. In contrast to bacteria, PMNs are thought to respond to a gradient through a spatial sensory mechanism rather than temporally (67-69). Zigmond and Sullivan (69) proposed that PMNs migrate to higher concentrations of a stimulant based on asymmetric distribution of chemotactic receptors on the leading edge of the phagocyte. As the receptors bind ligand at a particular concentration, an adaptive process may occur which renders the cell able to respond only to higher concentrations of stimulant. Mechanisms delineating the complex process of adaptation have not been fully elucidated. However, it is thought to be analogous to adaptation described in sensory systems.

Work from many laboratories indicates that functional responses can be modulated by a number of different mechanisms. In the late sixties, deactivation of PMN migration to serum factors was noted. Ward and Baker (70) found that preincubation of rabbit peritoneal exudate cells with activated rabbit serum inhibited migration in a dose and time related manner. Sullivan and Zigmond (71) later hypothesized deactivation was related to a loss of chemotactic receptor numbers (i.e., receptor downregulation) which occurred after exposure of PMNs to a chemotactic

factor. These investigators found pretreatment of PMNs from rabbit peritoneal exudates with N-formyl-l-norleucyl-l-leucyl-l-phenylalanine (FNLLP) decreased binding of FNLLP. They further showed the decrease in binding to be related to fewer binding sites available on the PMN membrane, as opposed to alterations in receptor affinity. Findings from other groups have further supported the relationship between receptor expression and functional responses. Vitkauskas et al. (72) showed a decrease in binding associated with decreased secretion responses. Donabedian and Gallin (73) reported decreased binding of FMLP associated with the transient preferential deactivation (homologous desensitization) seen in human PMNs after pretreatment with 1  $\mu$ M FMLP. However, it should be noted that the nonpreferential deactivation (heterologous desensitization) seen 60 minutes after treatment was marked by increased FMLP binding. Fluorescent probes have been utilized to generate data which further support the notion of ligand stimulated internalization of formylpeptide receptors. Niedel et. al. (74) showed, that as quickly as 2 minutes after exposure to a rhodamine labeled formylated peptide derivative, human PMNs exhibited receptor aggregation and internalization.

Other agents have been identified which cause receptor downregulation on myeloid cells (75-78). Treatment of HL-60 cells and K562 cells with PMA caused a reduction in transferrin receptors (75, 76). May et al. (78) hypothesized that PMA induced internalization is associated with hyperphosphorylation of the transferrin receptor. Andersson et al. (79) showed PMA to decrease specific binding of FMLP on human PMNs after receptors have been upregulated by increases in intracellular calcium.

Remarkably little is known about the fate of the receptor ligand complex once internalized. A model reviewed by Painter et al. (80)



suggests the following: FMLP binds to specific cell membrane receptors, the affinity of the receptor appears to increase after ligand has bound and the receptor then becomes associated with the cytoskeleton. This step is thought to be important for the subsequent internalization of the receptor-ligand complex. The receptor and ligand appear to dissociate at some point after internalization. In subcellular fractionation experiments, two fractions other than those of the plasma membrane bound FMLP. The more dense fraction has been reported to contain markers for Golgi as well as those for specific granules. It is assumed that receptors are eventually recycled to the cell surface since kinetic data indicate that sustained responses such as motility would require receptor replenishment.

Work by Gallin and coworkers (5) indicated pretreatment of PMNs with low doses of specific granule secretagogues such as PMA and A23187 resulted in increased binding and chemotactic response to FMLP. These investigators have hypothesized the association of intracellular formylpeptide receptor stores with specific granules after observing binding of FMLP to cellular fractions enriched in specific granules (5, 81-83). In support of this hypothesis are studies which showed that patients with specific granule deficiencies, as well as granule deficient cytodlasts, do not increase formylpeptide receptor expression after PMA pretreatment (84, 85). Evidence from other groups substantiates the existence of intracellular stores of formylpeptide receptors. Anderson and Nield (86) showed a bimodal distribution of intracellular pools of FMLP receptors in differentiated HL-60 cells using photoaffinity labeled FMLP. Jesaitis et al. (87) have suggested formylpeptide receptor pools are associated with the Golgi apparatus, an organelle thought to be involved in receptor recycling in a

number of receptor systems including transferrin, epidermal growth factor, and low density lipoproteins.

Other agents with perhaps more physiological relevance than PMA also have been shown to increase expression of formylpeptide receptors. One class of molecules with particularly exciting implications is the hematopoietic factors. Granulocyte-macrophage colony stimulating factor (GM-CSF), recently available as a recombinant molecule (rhGM-CSF), has been shown to enhance PMN responses to mediators such as  $LTB_4$ , FMLP and  $C5a_{des\ arg}$  (88). Weisbart et al. (89) reported pretreatment of PMNs with rhGM-CSF caused increases in formylpeptide receptor numbers as well as changes in receptor affinities. It has also been observed that in vitro pretreatment with lymphocyte inhibitory factor (LIF) caused an increase in formylpeptide receptor expression on human PMNs (90). LIF and rhGM-CSF appear to have similar effects on mature PMNs, while LIF lacks any colony stimulating activity. Goldman et al. (91) showed pretreatment of rabbits with lipopolysaccharide (LPS) caused a decrease in chemotaxis to  $LTB_4$  and  $C5a$  which appeared to be mediated by a decrease in binding of these two stimulants. Interestingly, migration to FMLP was not affected by the LPS treatment and there was close to a ten-fold increase in expression of formylpeptide receptors. Andersson et al. (79) have hypothesized that expression of formylpeptide receptors on the cell surface is related to intracellular levels of calcium. PMNs pretreated with intracellular calcium chelators and incubated for 60 minutes at  $37^{\circ}C$  did not increase formylpeptide receptor expression as did the cells with normal intracellular calcium. Additionally, restoration of normal intracellular calcium levels was associated with expression of increased numbers of formylpeptide receptors.



There may be significant physiological roles for increased formylpeptide receptor expression as well as changes in receptor affinity. As discussed, correlates have been observed involving increased receptor expression and increases in certain functional activities. However, receptor numbers are not always indicative of changes in functional responses as the LPS study by Goldman et al. (91) and the nonpreferential deactivation work by Donabedian et al. (73) illustrates. Cellular responses to mediators may be altered through changes in the coupling between the receptor, transducer and effector elements as well as biochemical events distal to the receptor.

## 2. Biochemical Modulation

PMA treatment has been shown to have dramatic effects on cellular parameters beyond those of receptor expression. Hoult and Nourshargh (92, 93) reported a 5 minute pretreatment with 10 nM PMA caused an enhancement of FMLP, PAF and  $LTB_4$  induced B glucuronidase and lysozyme secretion of human PMNs while the same conditions lead to decreased calcium mobilization. It was also observed that chemokinesis to the same ligands was decreased during a 4 hour continuous exposure to PMA. Receptor binding assays were not performed, however, the divergent effects of PMA on calcium mobilization versus degranulation argue that these actions occurred from changes distal to receptor occupancy. Naccache et al. (49) found elicited rabbit PMNs to have decreased release of B glucuronidase, as well as an impaired calcium mobilization, in response to FMLP and  $LTB_4$  after a 3 minute pretreatment with PMA at concentrations as low as 16 nM. This effect

was both concentration and time dependent. Lagast et al. (94) reported a similar effect of 100 nM of PMA on calcium response to FMLP in both human and guinea pig PMNs. Generation of  $IP_3$  by the chemoattractants FMLP,  $LTB_4$  and PAF is inhibited when human PMNs are pretreated with 160 nM PMA for 5 minutes (95). This supports the calcium mobilization data since  $IP_3$  is thought to be a primary stimulant for release of intracellular calcium stores. The causes for the discrepancies in the effects of PMA on degranulation are not readily apparent. Doses of 8 nM PMA had no effect on degranulation to FMLP in the studies of Naccache et al. (49). Gallin et al. (5) reported increases in lysozyme release by human PMNs migrating to C5a after a 30 minute pretreatment with doses of PMA ranging from 8 nM to 160 nM. Goldstein et al. (96), as well as O'Flaherty et al. (97) showed enhanced release of B glucuronidase and lysozyme after pretreatment with PMA. O'Flaherty (97) reported 5 and 50 nM of PMA to be a priming agent for degranulation by PAF,  $LTB_4$  and FMLP. Possible explanations for the PMA induced decreases in enzyme release seen by Naccache et al. (49) versus the increases found in other groups may relate to species differences in signal transduction paths between rabbit and human PMNs or differences in PMNs which have migrated to the peritoneum versus those of the peripheral blood. Both in vivo and in vitro work have established functional differences between those populations of PMNs which have migrated to stimulants (8, 98).

High concentrations of PMA have been shown to inhibit  $IP_3$  generation and subsequent calcium mobilization in other cell types. Pretreatment of an astrocytoma cell line with 1000 nM PMA inhibits the  $IP_3$  and calcium responses to a muscarinic agonist (48). Thrombin induced intracellular calcium increases in human platelets were also inhibited by



pretreatments with PMA at concentrations ranging from 20 to 1600 nM (50, 52).

LTB<sub>4</sub> has been shown to recruit human PMNs to respond to FMLP (99, 100). Gay et al. (100) reported that pretreatment with LTB<sub>4</sub> for 5 minutes caused no superoxide production yet significantly increased superoxide produced by FMLP. These conditions did not alter expression of formylpeptide receptors. Interestingly, there was no increase in response to opsonized zymosan or PMA. Fletcher (99), using nitroblue tetrazolium reduction to quantitate the oxidative burst, demonstrated an increase in FMLP induced membrane depolarization after LTB<sub>4</sub> pretreatment. PAF has also been shown to increase superoxide production in response to FMLP, as well as to PMA and opsonized zymosan (101, 102). Several mechanisms have been postulated to account for the priming seen in interactions between PMN stimulants. Increased intracellular calcium and ligand occupancy have a synergistic effect on PKC activation. This is illustrated most effectively in the synergism seen in degranulation stimulated by A23187 and PMA (49). Another possibility is the release of mediators such as LTB<sub>4</sub>, prostaglandins or other lipxygenase/cyclooxygenase derivatives by the priming agent which then act back on the PMN through yet another mechanism.

#### D. SPECIES SPECIFICITY OF FMLP RESPONSIVENESS BY PMNs

Reports from a variety of laboratories indicate an interesting distribution of species responsiveness to FMLP. Table 1 illustrates a summary of PMN functional responses to FMLP by various species.



TABLE I

SPECIES SPECIFICITY OF PMN  
RESPONSE TO FMLP

Responsive	Reference Citation	Poorly or Nonresponsive	Reference Citation
Human	3	Dog	12
Rabbits	7	Cow	14
Guinea Pigs	9	Pig	15
Rats	9	Cat	16
Horse	10	Sheep	15
		Horse	17
		Rhesus Monkey	108

Motility studies under agarose by Gray et al. showed cat (16) and cow (14) PMNs nonresponsive to FMLP as well as to the bacterial pentapeptide pepstatin. Additionally, cow PMNs did not migrate to filtrates from 7 different strains of bacteria which included E. coli (14). In a study comparing chemotaxis under agarose of peripheral PMNs from humans, rabbits, guinea pigs and pigs, Chenoweth et al. (15) found pig PMNs unable to respond to FMLP. The deficiency seen in the pig correlated with the lack of specific binding of tritiated FMLP.

Some conflicting data regarding horse PMN responsiveness to FMLP have been reported. Camp and Leid (17) demonstrated horse PMNs to be FMLP nonresponsive at concentrations ranging from 100  $\mu$ M to 1 fM in a modified Boyden chamber. However, Zinkl and Brown (10) showed chemotactic response of these cells, in an under agarose assay, at 100  $\mu$ M of FMLP. Snyderman and Pike (11) reported the lack of horse PMN chemotaxis to FMLP at concentrations ranging from 100  $\mu$ M to 1 pM in a modified Boyden chamber, finding degranulation at FMLP concentrations of 1  $\mu$ M.

Studies of dog PMNs have been conducted in several laboratories. Stickle et al. (13) noted dog PMNs did not polarize in response to FMLP at concentrations ranging from 1  $\mu$ M to 1 nM. Additionally, they failed to find specific binding of 50, 100, 150 and 300 nM tritiated FMLP. Redl et al. (12) saw no migration of dog PMNs to 1  $\mu$ M and 0.1  $\mu$ M of FMLP in leading front assays done in a Boyden chamber. These studies also showed no aggregation or receptors to FMLP. Interestingly, these cells both migrated and aggregated in response to dilutions of E. coli filtrate.

As discussed, physiological PMN stimulants can be divided into intrinsic and extrinsic sources. It appears from these studies that some



species have no capacity to respond to extrinsically generated stimulants. Cat PMNs did not migrate to FMLP or pepstatin, while cow PMNs did not migrate to FMLP, pepstatin or bacterial filtrates (14, 16). Based on these results the authors concluded neither of these two species could respond to extrinsic mediators. The work in pigs did not include pepstatin or bacterial filtrates (15). It is of interest that dog PMNs responded to E. coli filtrates while not responding to FMLP (12). It is possible other entities in the filtrates were responsible for the aggregation and motility observed or some component in the filtrate primed the PMNs so they subsequently became responsive to FMLP. Nonresponsiveness to FMLP has been interpreted as resulting from an absence of receptors for the molecule. This hypothesis has been supported by the lack of specific binding of tritiated FMLP by nonresponsive species such as the pig and dog (12, 13, 15). No literature exists assessing the possibility of converting a FMLP nonresponsive species responsive by pretreatment with agents capable of priming the cell through either modulation of receptor expression or through amplification of biochemical signaling paths. Previous work (83, 86, 87) confirms the existence of intracellular receptor pools for FMLP in responsive species. No work to date has examined the possibility that these pools exist and can be expressed in nonresponsive species, under appropriate conditions.

Using the promyelocytic cell line HL-60 it has been shown that formylpeptide receptors are expressed on approximately 20-40% of the cells during DMSO induced differentiation (103). During the course of differentiation, these cells acquired the ability to adhere, migrate, reduce NBT and phagocytize (103). These studies established functional parameters of differentiation followed morphological differentiation in this



model. Recent work by Sullivan et al. (104, 105) further supported this differentiation model, correlating development of functional responses to FMLP with acquisition of formylpeptide receptors in normal human bone marrow induced with rhGM-CSF. Chaplinski and Niedel (106) found treatment of HL-60 cells with dibutyryl adenosine 3'5'-cyclic monophosphate (dbc AMP), for 2 hours, stimulated formylpeptide receptor expression and 24 hour treatment induced receptors in 95% of the cells. Receptor expression was inhibited by cyclohexamide and therefore dependent upon protein synthesis. Increased receptor expression was also dependent upon continuous exposure of the cells. Removal of dbcAMP after 24 hours resulted in only 16% of the cells expressing the formylpeptide receptor at 72 hours. This work did not clarify whether observed receptor losses were caused by cessation of receptor synthesis and subsequent degradation or internalization of the receptors on the cell membrane. Interestingly, it was observed that functional differentiation assessed by adherence and NBT reduction was not complete until the cells had been exposed to the inducer for 72 hours, thus establishing a model divorcing formylpeptide receptor expression from the functionally mature phenotype. Treatment with compounds which raise cAMP such as  $\text{PGE}_2$ , theophylline and cholera toxin produced similar divergence of formylpeptide receptor expression and functional maturity (106).

The horse PMN has been shown to be an interesting model for divergent signaling paths in stimulus response coupling of PMNs. The data of Snyderman and Pike (11) demonstrate very few numbers of formylpeptide receptors on the horse PMN versus those of more responsive species (630 compared to 25,000 or more depending on the laboratory and species). It was suggested by the authors that so few receptors may not have the

capacity to trigger and maintain motility since this is a sustained response thought to require receptor recycling and replenishment. Small numbers of receptors, or those of very low affinity, may exist on the membrane of nonresponsive species and yet are unable to trigger significant responses. Priming of these cells may amplify signals by this otherwise hidden population of receptors over some physiological threshold and result in detectable responses to FMLP.

Generation of chemotactic and chemokinetic motility, calcium mobilization from intracellular as well as membrane stores, secretion of both primary and secondary granules and production of reactive oxygen intermediates can all be triggered in the PMN through receptor mediated processes. The regulation and appropriate elicitation of these events are of crucial importance to the host. Study of species differences in FMLP responses could lead to important information leading to a more thorough understanding of basic PMN physiology. The work in horse PMNs illustrates differences in stimulus response coupling demonstrated through such comparative studies. Experimentation capitalizing on naturally occurring diversity among species in response to FMLP may provide valuable information pertinent to enormously complicated mechanisms of stimulus response coupling in the PMN.



## II. RATIONALE

These studies were based on the hypothesis that the PMNs of species poorly or nonresponsive to FMLP, such as the dog or the rhesus monkey, can be induced to respond to FMLP by pretreatment with an appropriate priming agent. This hypothesis predicts that the inability to respond to FMLP is phenotypic as opposed to genotypic. Objectives were to determine whether treatment with pharmacological compounds such as PMA, as well as physiological mediators such as rhGM-CSF, enhance PMN responses to the chemoattractant FMLP in dogs and rhesus monkeys. Functional responses evaluated were migration and generation of the oxidative burst. The hypothesis was tested in an in vitro system with both dog and monkey PMNs and in vivo in monkeys. Dog PMNs were pretreated with PMA in vitro and tested for migration to FMLP. Studies utilizing rhGM-CSF employed PMNs from normal rhesus monkeys. RhGM-CSF has been shown to be species specific. The rhesus monkey was chosen as a model for the rhGM-CSF studies because, in contrast to the dog, monkey hematological responses to rhGM-CSF have been well documented.

The species specificity of PMN responsiveness to FMLP, an extrinsic mediator of inflammation, has previously been recognized. If a species thought to be FMLP nonresponsive can be induced to respond to this molecule, then this could provide a valuable model for the study of chemotactic receptor modulation or for addressing questions such as the role of PKC and cGMP activated protein kinase in PMN stimulus-response coupling.



### III. EXPERIMENTAL DESIGN

#### A. COMPARATIVE STUDIES

Prior to addressing the effects of PMA and rhGM-CSF on dog and monkey PMN responses to FMLP, studies were conducted to compare species responsiveness to FMLP in PMNs of dogs, monkeys and humans. A survey of data from a number of laboratories indicated wide variability in results obtained from group to group. Investigators have reported that dog PMNs do not polarize, migrate or aggregate in response to FMLP, observations supported by an absence of binding of  $^3\text{H}$ -FMLP to dog PMNs (12, 13). Reports from several laboratories have shown that rhesus monkey PMNs are responsive to FMLP although less so than the human PMN (107, 108).

To confirm these observations in our assay systems, as well as further expand these data, migration and generation of the oxidative burst to FMLP were compared between rhesus monkeys, dogs and humans. Studies in human PMNs were included to assure procedures utilized for this project generated results comparable to those reported in the literature, in addition to serving as positive controls for the nonresponsive species. Dose response curves for migration of PMNs from each species were generated for comparison of optimal FMLP concentrations, maximal numbers of cells migrating as well as a comparison of stimulation ratios (see Materials and Methods Chapter, Section K for definition). Studies were performed evaluating percentages of nitroblue tetrazolium (NBT) responsive PMNs from humans, rhesus monkeys and dogs in the presence and absence of FMLP. The results of these functional studies were supplemented by

studies assessing the expression of formylpeptide receptors on the membrane of PMNs from each of the species of interest.

## B. DOG STUDIES

### 1. Modulation of Dog PMN Responses to FMLP

#### a. Overview

The purpose of these studies was to determine if dog PMNs could be induced to migrate to FMLP after pretreatment with PMA or A23187 and to determine if these effects were accompanied by expression of formylpeptide receptors on the dog PMN membrane. Tremendous advances in understanding receptor mediated endocytosis have been made through the use of mutants with defective receptors as in the LDL receptor system (109). It was important to determine if dog PMNs had deficiencies in receptor expression which could be reversed. Identification of a block in formylpeptide receptor expression would be invaluable in future studies pertaining to the elucidation of structural domains or biochemical events such as phosphorylation important in chemotactic receptor recycling.

#### b. PMA Modulation of Dog PMN Responses to FMLP

Dog PMNs were pretreated with low concentrations of PMA and assessed for migration to FMLP and expression of formylpeptide receptors. Enhanced functional responses by human PMNs after pretreatment with 0.1 to 1 nM PMA have been reported by several groups (5, 97). It was previously suggested that migration increases result from mobilization of receptor stores associated with specific



granules (83). PKC has also been shown to regulate receptor affinity for ligand (110).

c. A23187 Modulation of Dog PMN Responses to FMLP

A23187 is a calcium ionophore which elicits specific granule release at low concentrations and is a complete secretagogue at higher concentrations in both human and dog PMNs (111, 112). Dog PMNs were pretreated with A23187 and assessed for migration to FMLP. These studies were important in discerning if mobilization of intracellular receptor stores were involved in enhancement of dog PMN function by PMA or if other responses resulting from PKC activation by PMA were involved.

d. Effects of n-Butanol on Binding of FMLP by Dog PMNs

Reports by two laboratories indicated that exposure of PMNs to n-butanol caused increased specific binding of tritiated FMLP (61, 62). It was postulated that this aliphatic alcohol caused increases in membrane fluidity resulting in expression of cryptic receptor populations in the membrane. Based on these reports the effects of n-butanol on binding of FMLP to dog PMNs were tested to determine if such cryptic receptor populations were present. Binding assessment was chosen as a screening measure instead of migration because the effects of n-butanol on FMLP binding appear quite readily while increases in motility were related to changes in optimal times to evaluate migration.



## 2. Dog PMN Migration to E. coli Filtrates and Formylpeptide Analogs

### a. Overview

Independent reports from 2 laboratories indicated that dog PMNs migrated to E. coli filtrates (12, 113). It was unknown if the migration stimulated by these preparations resulted from FMLP present in the supernatants, if a component such as LPS in the preparation primed the PMNs to respond to FMLP, if other formylated peptide molecules were present which had chemotactic activity or if an entirely different component of the filtrate caused the migration. Studies were undertaken to confirm work demonstrating E. coli filtrates as an effective dog PMN chemoattractant and determine if migration was elicited through the formylpeptide receptor.

### b. Migration of Dog PMNs to E. coli Filtrates

E. coli filtrates were prepared as described in the original work of Schiffmann et al. (1). These filtrates were tested as stimulants for migration using human PMNs and found to be effective at dilutions ranging from 1/500 to 1/10. Studies were then performed using dog PMNs to assess if these filtrates stimulated motility. As reported by others (12, 113), E. coli filtrates were very potent stimuli for dog PMN migration (See Figure 20, page 108 of the Results Chapter for the data).

### c. Signal Transduction Studies

Studies were initiated to determine if dog PMN migration to the filtrates was inhibited by PT. G regulatory proteins have been identified as transducer elements in the signal transduction of numerous cell-receptor systems. Recent work has identified G protein involvement

in the generation of many PMN functions. Pertussis toxin ADP ribosylates the alpha subunit of  $G_c$  and  $G_i$ , 2 members of the family of G regulatory proteins (114). Many laboratories have demonstrated that functional responses such as chemotaxis, degranulation and the oxidative burst of PMNs in response to  $LTB_4$ , FMLP, PAF and others are inhibited by pretreatment with pertussis toxin (115). ADP ribosylation results in an effective uncoupling of the chemotactic receptor from PLC. Inhibition of migration by PT would suggest a receptor coupled response to E. coli filtrates.

#### d. Studies with Formylpeptide Receptor Antagonists

Inhibition of migration to E. coli filtrates with a formylpeptide receptor antagonist would discern if migration is elicited specifically through the formylated peptide receptor. Control experiments were first performed documenting the inhibition of human PMN migration to FMLP using the antagonist t-boc-MLP. The affinity of antagonists available for the formylpeptide receptor is quite low, the  $ID_{50}$  of t-boc-MLP is 60  $\mu$ M in rabbit PMNs (116) in contrast to an  $ED_{50}$  for rabbit PMN migration to FMLP of 70 pM (1). Results of studies in humans indicated that PMN migration to FMLP could be inhibited by t-boc-MLP although an excess of antagonist 10,000-fold greater than agonist was necessary (See Figure 22, page 112 of the Results Chapter for the data). Results of these dog PMN studies substantiated further investigation into responses mediated through the formylpeptide receptor.



### 3. Responses of Dog PMNs to High Concentrations of FMLP

#### a. Overview

The next series of experiments examined the possibility that dog PMNs would migrate to high concentrations of FMLP. Studies described in the comparative section did not examine migration to FMLP at concentrations higher than 1  $\mu$ M. The original decision not to test higher concentrations of FMLP was based on results from studies of human and rabbit PMNs where the  $ED_{50}$  was identified in the picomolar range and maximum migration was found at FMLP concentrations between 1 and 100 pM (3, 8).

Indirect and direct lines of evidence point to possible differences in existing formylpeptide receptors among species. Snyderman and Pike demonstrated that horse PMNs do not migrate to FMLP although these cells degranulate and possess several hundred high affinity receptors (11). Zinkyl and Brown reported that horse PMNs migrated to 100  $\mu$ M FMLP under agarose (10). Work by Askamit and Harvath suggested species differences between structure activity relations of formylpeptide receptors in humans, guinea pigs and mice (117). These studies prompted the examination of the capacity of dog PMNs to respond to high concentrations of FMLP.

#### b. Migration of Dog PMNs to High Concentrations of FMLP

Experiments were performed assessing migration of dog PMNs to 0.01, 0.1 and 1 mM of FMLP. Chemotactic and chemokinetic components of migration were delineated. Subsequent work addressed the role of a specific receptor in these responses through utilization of PT. Unfortunately, FMLP antagonists could not be used to further



support the specificity of the response because the concentration of the agonist necessary to stimulate migration far exceeded the  $IC_{50}$  of the antagonist.

#### c. Generation of the Oxidative Burst by Dog PMNs

Studies in FMLP responsive species indicate migration is elicited at low concentrations of stimulant and production of reactive oxygen intermediates occurs at higher concentrations. The transduction of the signal for these divergent functions is currently an area of intensive research. To further explore the unusual dog response to FMLP, studies were performed assessing generation of the respiratory burst using the nitroblue tetrazolium assay (NBT). PMNs were exposed to RPMI 1640 (a media), 0.01 to 1 mM FMLP or FMLP in the presence of cytochalasin B. Cytochalasin B is an extraction from fungus which has been shown to enhance the effects of receptor mediated ligands in the absence of adherence (118). The mechanism of action is largely unknown. However, this molecule has been used by many laboratories to increase both the oxidative burst and degranulation in response to receptor mediated stimulants. Percentages of NBT positive cells were assessed in FMLP stimulated cultures and compared to media controls.

### 4. Responses of Dog PMNs to Formylpeptide Analogs.

#### a. Studies of Dog PMN Response to Formylpeptide Analogs

A variety of formylated peptide analogs are available to evaluate structure activity relations of the formylpeptide receptor (1, 2, 116, 119, 120). The migration of dog PMNs to high concentrations of FMLP (See Figure 24, page 117 of the Results Chapter for the data) in the

absence of detectable  $^3\text{H}$ -FMLP binding indicated either low affinity or low numbers of formylpeptide receptors on the plasma membrane. Studies were directed towards identification of some high affinity analogs to which dog PMNs would migrate and determination of chemotactic or chemokinetic components of that migration. Pertussis toxin pretreatment was performed to assess if these responses were receptor mediated. Again, the formylpeptide receptor antagonists could not be utilized effectively in these studies due to the high  $\text{IC}_{50}$ .

### C. RHESUS MONKEY STUDIES

#### 1. Rationale

The dog studies established that migration to FMLP could be enhanced with PMA pretreatment and that formylpeptide receptors of either low affinity or low numbers existed on the membrane of the dog PMN. It was of interest to attempt to work with conditions more similar to possible physiological events. PMN responses are modulated by many inflammatory mediators.  $\text{LTB}_4$  enhanced PMN production of reactive oxygen intermediates in response to FMLP through modification of events distal to receptor binding (99). PAF increased PMN responses to FMLP both in vivo and in vitro (101, 102). RhGM-CSF primed responses to FMLP through upregulation of chemotactic receptor number (89), alterations in receptor affinity (89) and amplification of the calcium signal (121).

The rhesus monkey was chosen to evaluate rhGM-CSF mediated modulation of PMN migration to FMLP in a species poorly responsive to FMLP. GM-CSF is a species specific molecule (122) but, recombinant



human GM-CSF (rhGM-CSF) has been shown to be active in monkeys (123). Hematopoietic parameters of rhesus and cynomolgous monkeys increased dramatically after in vivo administration of rhGM-CSF (123). An advantage of utilizing rhGM-CSF for these studies was the biological origin of the molecule allowing either in vivo or in vitro administration. Preliminary evaluation of rhesus monkey PMN migration to FMLP established the poor response by this species. Low percentages of rhesus monkey PMNs migrated to FMLP and at concentrations quite different from human PMNs (See Figures 10 and 11, pages 82 and 84 of the Results Chapter for the data). These studies sought to determine if rhGM-CSF enhanced rhesus monkey PMN responses to FMLP and to investigate if this was mediated through receptor upregulation. PMNs were treated with rhGM-CSF both in vivo and in vitro to address these questions. The in vitro experiment determined if increased FMLP responses found in human PMNs could be reproduced in PMNs from the rhesus monkey. The in vivo studies provided the novel opportunity to assess how these effects would translate into the multicomponent system operative systemically.

The following sections will describe studies addressing rhGM-CSF as a priming agent.

## 2. In Vitro Modulation of Rhesus Monkey PMN Functional Responses

Studies were conducted to determine the effects of in vitro pretreatment with rhGM-CSF on migration and oxidative burst responses of isolated monkey PMNs to FMLP. In the human, in vitro pretreatment with rhGM-CSF has been shown to enhance both migration and the oxidative



burst to FMLP (89). An interesting relationship has been identified between the time required for enhancement of migration versus the oxidative burst (89). Short exposures to rhGM-CSF (5 to 15 minutes) increased motility while longer exposures of 90 to 120 minutes were necessary to enhance the oxidative burst. Both events are associated with upregulation of the formylpeptide receptor in human PMNs (89). This chronological relationship was further explored with the rhesus monkey PMN. Experiments were also performed to determine if in vitro treatment with rhGM-CSF caused an increase in binding of FMLP.

Additional studies were conducted to further clarify the actions of rhGM-CSF in amplifying the inflammatory response to FMLP. Examples of agents modulating PMN responses have been well documented in the literature. The terms priming and recruitment are often used interchangeably when referring to enhancement of responses to a particular stimuli. For the purposes of this project, recruitment will be defined as conversion of a nonresponsive PMN to a responsive state and priming as an enhancement in function of a previously responsive PMN. The motility assay used for this project effectively measures cell numbers migrating which is easily converted to percentages of responsive cells. Therefore, an increase in cells migrating can be interpreted as recruitment. The NBT assay determines increases in percentages of cells responding (i.e., recruitment). Additionally, by grading intensity of positive cell staining and calculating a weighted average for stain intensity, a qualitative measure of reactive oxygen intermediate production can be generated so that priming effects are assessable.

Peripheral blood was drawn from normal monkeys, PMNs isolated and exposed to appropriate concentrations of rhGM-CSF for time periods

ranging from 5 to 120 minutes. Migration, NBT, dichlorofluorescein (DCF) and binding studies were performed as described in the Materials and Methods Chapter.

### 3. In Vivo Modulation of Rhesus Monkey PMN Functional Responses

Studies were performed to determine if treatment of monkeys with rhGM-CSF in vivo increased migration to FMLP and PAF. Work by Mayer et al. had shown in vivo treatment of normal rhesus monkeys with rhGM-CSF increased PMN superoxide production in response to FMLP as well as increased killing of E. coli (124). No reports had described the in vivo effects of rhGM-CSF on migration. An investigation of the effects of in vivo administration of rhGM-CSF on PMN function is important for several reasons. First, it addresses the role this molecule may play in a more physiological situation than a test tube can provide. Secondly, enhancement of FMLP responsiveness may occur through direct actions of rhGM-CSF on the PMN, as suggested by the in vitro work, through indirect means including alterations during maturation of the PMN in the marrow or through triggering the release of other mediators. In vivo studies are necessary to resolve these issues. These studies are also significant since rhGM-CSF is being utilized in patients with AIDS and myelodysplastic syndrome as well as in patients neutropenic after chemotherapy (125, 126). The potential of this molecule to accelerate PMN recovery in vivo and its capacity to enhance the functional properties of PMNs makes studies of the effects of rhGM-CSF on PMN function important.



Monkeys were administered 50,400 U/Kg/day of rhGM-CSF for 7 days using an osmotic minipump. Peripheral blood was collected on days 0, 2, 5, 7, 9, 12, 16, 21 and 28. PMNs were purified as described in the Materials and Methods Chapter. Motility parameters of interest included random migration as well as total migration and chemokinesis to FMLP and PAF. Binding studies were performed (when possible) on monkeys to assess changes in formylpeptide receptor expression.

Preliminary studies indicated that in vivo administration of rhGM-CSF to normal rhesus monkeys increased migration to FMLP on days 5 and 7 of factor treatment (See Figure 37 A, page 151 of the Results Chapter for the data). The delay in enhanced migration stood in apparent contrast to the rapid effects of rhGM-CSF on FMLP responses found in the in vitro studies (See Figure 30, page 133 of the Results Chapter for the data). Experiments were undertaken to evaluate the capacity of PMNs from rhesus monkeys treated with rhGM-CSF for 5 days to be further recruited in vitro.

## IV. MATERIALS AND METHODS

### A. PMN SOURCES

#### 1. Dogs

Healthy, pure-bred male and female beagles (9-12 kg) from Hazelton Laboratories were used for these studies. They were housed in temperature controlled rooms within individual stainless steel cages at AFRRI, an AALAC accredited animal facility. The dogs were fed kibbled laboratory dog food, supplemented once a week with high protein canned meat ration. Water was provided ad libitum. Peripheral blood was drawn with 19-21 gauge needles from the cephalic vein in the foreleg. Preservative free heparin was used as an anticoagulant at 10 U/ml. Blood drawing was done by a team of ALAAS certified technicians. Previous experience had shown it to be tolerated without evidence of pain or discomfort by the animal.

#### 2. Rhesus Monkeys

Domestic born male rhesus monkeys, (Macaca mulatta) with a mean weight of 4 Kg were used in these studies. They were housed in individual stainless steel cages in conventional holding rooms at AFRRI. Monkeys were provided with commercial primate chow supplemented with fruit and tap water ad libitum. Peripheral blood was drawn using a 19-21 gauge needle from the saphenous vein of monkeys sedated with ketamine



hydrochloride, (10 mg/kg, intramuscularly). Preservative free heparin was used as an anticoagulant at 10 U/ml.

All research was conducted according to the principles enunciated in the "Guide for the Care and Use of Laboratory Animal Resources", National Research Council.

### 3. Humans

Buffy coats were collected by the NIH blood bank utilizing standard procedures. Briefly, 500 ml of peripheral blood were collected from normal human donors into Fenwal collection bags containing 63 ml of CPDA (citrated phosphate with dextrose and adenine, prepared with 2 g dextrose, 1.66 g sodium citrate, 206 g of citric acid, 140 mg of sodium phosphate monobasic and 17.3 mg of adenine per liter of distilled water). The blood was then centrifuged 2500 rpm for 3 minutes. White blood cells (wbcs) layered over the red blood cell (rbc) pellet and were collected with a blood extractor. These wbcs were placed in a Fenwal bag and stored at room temperature until use. All donors used for these studies were screened for HIV antibodies and hepatitis B antigen.

### B. PMN SEPARATION TECHNIQUES

Several methods of preparing purified PMNs were utilized in these studies. The following is a description of each.

## 1. Dual Discontinuous Percol Gradients

PMNs were separated as described by Thomas et al. (127). Peripheral blood was washed once in Hank Balanced Salt Solution without  $\text{Ca}^{++}$  or  $\text{Mg}^{++}$  (HBSS w/o). The packed cellular elements were diluted with one volume of HBSS w/o (Gibco) and layered over dual discontinuous percol (Pharmacia) gradients of densities 1.077 g/ml and 1.119 g/ml. This preparation was centrifuged at 2000 rpm for 30 minutes at room temperature. Purified PMNs were collected from the interface between the 1.077 and 1.119 density layers of percol. These cells were washed once in 5 to 10 volumes of HBSS w/o. Rbcs were lysed in 0.15 M ammonium chloride buffer (8.2 g ammonium chloride, 1 g potassium carbonate and 29 mg of EDTA in 1 liter of distilled water) and the resultant preparation was washed twice in HBSS w/o. PMNs were resuspended in an appropriate media, cell counts performed on a hemocytometer and slides prepared for differentials. This was the means of isolating dog PMNs except where indicated.

## 2. Dual Ficoll Gradients

PMNs were prepared utilizing a modification of the method described by English and Anderson (128). Separations were performed as described for the percol separations except ficoll (density 1.119-Sigma Chemical, density 1.077-Bionetics) was the separation medium



### 3. Ficoll Followed by Dextran Sedimentation

This separation was performed with modifications of the method published by Boyum (129). Peripheral blood was washed in HBSS w/o. Packed cellular elements were resuspended in 1 volume of HBSS w/o and layered over ficoll of density 1.077 g/ml (Bionetics). This preparation was centrifuged at 2000 rpm for 30 minutes at room temperature. PMNs were collected from the top of the rbc pellet. These cells were washed once in HBSS w/o, resuspended in one volume of 6% dextran (Pharmacia) solution and allowed to sediment for 45 minutes at room temperature. The supernatant was collected, spun down and remaining rbcs lysed with 0.15 M ammonium chloride buffer. These cells were then washed twice in HBSS w/o and resuspended in appropriate media. Cell counts were then performed on a hemocytometer and slides prepared for differentials.

### 4. Dextran Sedimentation

Peripheral blood was washed in HBSS w/o. Packed cellular elements were resuspended in 1 volume of HBSS w/o and diluted with 1 volume of 6% dextran in a modified version of that described by Boyum (129). The cell suspension was then incubated 45 minutes at room temperature. The supernatant was collected, spun down and rbcs lysed with ammonium sulfate as previously described above. Resulting PMNs were washed twice in HBSS w/o and resuspended in appropriate media. Cell counts were performed on a hemocytometer and slides prepared for differentials.

## 5. Whole Blood Lysis

Peripheral blood was diluted with 5 to 10 volumes of 0.15 M ammonium chloride buffer. After an incubation period of approximately 10 to 15 minutes the cells were washed twice in HBSS w/o, resuspended in an appropriate media and counted. Slides for differential counts were prepared as well. Except where indicated this was the means which was used to isolate monkey PMNs.

## 6. Ficoll Followed by Lysis

Peripheral blood was washed in HBSS w/o. Packed cellular elements were resuspended in 1 volume of HBSS w/o and layered over ficoll of density 1.077 g/ml. This preparation was centrifuged at 2000 rpm for 30 minutes at room temperature. PMNs were collected from the top of the rbc pellet. These cells were washed once in HBSS w/o and then rbcs lysed as described in the percol gradient section. Slides for differential counts were prepared. Except as indicated this is the method which was utilized to isolate human PMNs.

## C. PRIMING STUDIES

### 1. In Vitro Priming of Dog PMNs With PMA

Purified PMN suspensions from dog peripheral blood were resuspended at  $10^6$  cells/ml in the media RPMI 1640 (Biofluids) with 0.1, 1 or 10 nM of PMA (Sigma). Cells were incubated at 37° C for 5



minutes. Cells were then spun down, supernatant discarded and resuspended in RPMI 1640, 1% fetal calf sera (fcs) for motility studies.

## 2. In Vitro Priming of Dog PMNs with A23187

Purified PMN suspensions from dog peripheral blood were resuspended at  $10^6$  cells/ml in RPMI 1640 (Biofluids) with 1, 10 or 100 nM of A23187 (Sigma). Cells were incubated at  $37^{\circ}$  C for 30 minutes. Cells were then spun down, supernatant discarded and resuspended in RPMI 1640, 1% fcs for motility studies.

## 3. In Vitro Priming of Rhesus Monkey PMNs with rhGM-CSF

In vitro priming studies with rhGM-CSF (Genetics Institute) were conducted using methods published by Weisbart et al. (89). In brief, PMNs were incubated with rhGM-CSF ranging from 10 to 1000 pm (1 to 100 U/ml) for 0, 5, 15, 60 and 120 minutes. Cells were then assessed for migration to the stimulants FMLP (Sigma) and PAF (Sigma) or for generation of the oxidative burst to FMLP using the NBT and DCF assay.

## 4. In Vivo Priming of Rhesus Monkey PMNs with rhGM-CSF

### a. RhGM-CSF Administration by Osmotic Minipump

Functional responses of peripheral PMNs from monkeys receiving rhGM-CSF in vivo utilizing an osmotic minipump were evaluated. Peripheral blood was collected prior to pump implantation 2 to 4 times in order to establish baseline values for functional parameters.

Pumps were implanted on day 0 and removed on day 7. Peripheral blood was collected for evaluation on days 0, 2, 5, 7, 9, 12, 16, 21 and 28. PMNs were collected using the whole blood lysis procedure described. Alzet miniosmotic pumps (#2ML1, ALZA corp, Palo Alto, CA), delivering 50,400 units/kg/day of rhGM-CSF over a period of 7 days, were used in these experiments. For surgical implantation of the pumps, the monkeys were administered 4% sodium thiamylal (10 mg/kg, intravenously). Alzet pumps, filled with the appropriate concentration of rhGM-CSF or saline, were implanted subcutaneously along the midline of the back, caudal to the scapular crests. The skin was closed with 4/0 chromic gut placed in a continuous subcuticular suture pattern and bandaged. These procedures were performed by the staff veterinarian at AFRRI. Monkeys were initially conditioned to wearing rhesus monkey jackets (Alice King Chatham, Los Angeles, CA), then were re-jacketed after the pump was implanted to protect the pump site and were worn continuously for the next 21 days. At the end of 7 days, the pump was removed through the original surgical incision; the site flushed with a 50:50 hydrogen peroxide-sterile water solution then betadine; packed with betadine soaked gauze; then lightly bandaged. The packing was changed on a daily basis for the next several days and continuously treated until healing was complete. All pump implantation sites healed within 7 days after pump removal. The pump site was cultured for bacteria after pump removal. The monkeys were checked twice a day during the period of experimentation by certified animal technicians.



#### b. RhGM-CSF Administration by Subcutaneous Injection

Monkeys received subcutaneous injections twice daily of either rhGM-CSF at 15,000 U/kg/day (n=2), 50,000 U/kg/day (n=1) or saline. PMNs isolated from these animals after 5 days of rhGM-CSF treatment were exposed in vitro to 10 U/ml rhGM-CSF for 0, 5, 15, and 60 minutes and then assessed for migration to 0.1  $\mu$ M FMLP.

#### D. EFFECTS OF n-BUTANOL ON EXPRESSION OF FORMYLPEPTIDE RECEPTORS BY DOG PMNs

Purified PMN suspensions from dog peripheral blood were resuspended at  $10^7$  cells/ml in HBSS w/o (Gibco) with 2.5% or 0.25% n-butanol. Cells were incubated for 15 minutes at room temperature and then assessed for expression of formylpeptide receptors.

#### E. MOTILITY STUDIES

##### 1. Overview

Direct microscopic observation, migration under agarose and filter assays are three major means with which to quantitate motility of leukocytes in vitro (130). Migration through cellulose ester filters, originally described by Boyden (131), revitalized study of leukocyte locomotion by providing a relatively simple means by which to quantitate cell numbers migrating. Distance migrated by the leading edge of PMNs within the filter was later introduced as another measure of motility (130). In 1980, Falk et al. described a new filter assay using a 48 well chemotactic

chamber with polycarbonate filters and 5  $\mu$ M pores (132). Key advantages of this assay compared to the cellulose ester filter assay are the few cell numbers necessary for a given test, excellent reproducibility as well as rapid quantitation of cell numbers. Additionally, cells are in the same focal plane and morphology is excellent.

## 2. Migration Methodology

PMNs separated through one of the described procedures were assessed in chemotaxis assays using a modified version of the methods reported by Falk et al. (132). Chemoattractant or media (26  $\mu$ l) was seeded in the lower compartment of a 48 well chemotactic chamber (Neuro Probe). Polycarbonate filter paper (25 mm X 80 mm, 10  $\mu$ M thick with 5  $\mu$ M pores and polyvinylpyrrolidone free, Neuroprobe) was placed on top of the wells so contact was made with the contents of the lower wells. A gasket and the upper portion of the chamber was then affixed over the filter strip and the assembly incubated 30 minutes in a humidified incubator maintained at 37° C and 5% CO<sub>2</sub>. PMNs were resuspended in RPMI 1640 and 1% fcs at the cell concentration determined to be optimal for the species being tested. This cell preparation (45  $\mu$ l) was added to each of the upper compartments of the chamber and incubated for 60 minutes or whatever time period appropriate for the experiment. The filter strips containing migrating cells were harvested as follows. The filter strip was removed from the chamber with the side containing cells which had migrated upwards. A clamp was placed on each end of the strip and the nonmigrating side gently scraped to remove the cells. The strip was fixed for 1 minute in methanol and stained in Dif-Quick. Numbers of cells



migrating were assessed with an Optimax Image Analyzer attached to a Leitz microscope equipped with a Chalnicon television camera.

### 3. Chemokinesis

Studies to assess which component of migration to stimulants was chemokinetic were performed as described for chemotaxis except cells were exposed to uniform concentrations of a stimulant as opposed to a gradient. This method was described by Boyden (131) and has been routinely used in polycarbonate filter assays to quantitate chemokinesis.

### 4. Adherence

To evaluate total numbers of migrating PMNs, determinations were made of numbers of cells which fell into the lower well as well as numbers of cells which migrated and then remained attached to the polycarbonate filter paper. This was done by collecting the contents of the lower well of the chemotactic chamber at the end of a motility assay and performing cell counts.

### 5. Preparation of Chemoattractants

All chemoattractants were prepared from freshly thawed aliquots of stock solutions on a given test day.

#### a. Monkey ZAS

Serum from a normal monkey was incubated for 60 minutes with 15 mg/ml of Zymosan (Sigma) while rotating end over end. Serum

was then heat inactivated for 30 minutes at  $56^{\circ}$  C, spun at 1000 X g for 15 minutes at  $4^{\circ}$  C, aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

b. E. coli Filtrate

Human E. coli strain (AFFRI 6) was grown overnight in 50 mls of RPMI 1640. The suspension was centrifuged at 3000 rpm,  $4^{\circ}$  C and the supernatant removed. The supernatant was filtered through a 0.45 uM millipore filter, aliquoted and frozen at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

c. FMLP

A 0.1 M stock solution of FMLP (Sigma) was prepared in DMSO (Fisher), aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

d. PAF

A 0.1 uM stock solution was prepared in RPMI 1640. The stock solution was aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

e. FMLPP

A 0.01 M stock solution of FMLPP (Sigma) was prepared in DMSO (Fisher), aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

f. FMLPB



A 0.01 M stock solution of FMLPB (Sigma) was prepared in DMSO (Fisher), aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

g. FNLLPNLTL

A  $2 \times 10^{-4}$  M stock solution of FNLLPNLTL (Sigma) was prepared in DMSO (Fisher), aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

h. t-boc-MLP

A 0.1 M stock solution of t-boc-MLP (Sigma) was prepared in DMSO (Fisher), aliquoted and stored at  $-70^{\circ}$  C. On each test day an aliquot was thawed and diluted to the appropriate concentration in RPMI 1640.

## F. OXIDATIVE BURST STUDIES

### 1. Overview

Assessment of the oxidative burst generation was performed using the nitroblue tetrazolium assay (NBT). NBT is a soluble yellow redox dye which is taken into the cell cytoplasm. In the presence of reactive oxygen intermediates produced during the oxidative burst, NBT is reduced to formazan, an insoluble compound which precipitates in the cell cytoplasm. Percentages of NBT positive cells are assessed. One advantage of this assay relevant to the project was the ability to evaluate degree of formazan

deposition in individual cells. This assay is an easy and rapid means with which to address NADP oxidase activation.

## 2. Nitroblue Tetrazolium (NBT) Assay

A 0.4% stock solution of NBT (Sigma) was prepared and frozen in aliquots at  $-70^{\circ}$  C. Immediately prior to use the stock was thawed, diluted with 1 volume of RPMI 1640, 5% dextrose (Gibco) and sterilized through a 0.45  $\mu$ M Millipore filter. RPMI 1640 or FMLP (50  $\mu$ l) as well as 50  $\mu$ l of RPMI 1640 or cytochalasin B (Sigma) were added to the NBT preparation. PMNs (0.2 ml) at  $4 \times 10^6$ /ml in RPMI 1640 were added to 0.2 ml of the above preparation and incubated 15 minutes at  $37^{\circ}$  C. The samples were then centrifuged 10 minutes, resuspended and slides prepared. Slides were stained 1 minute in methanol, 3 minutes in 0.4% safranin-O (Sigma) and air dried. After mounting cover slips a minimum of 100 PMNs were assessed for formazan granule deposition and scored as percentages of NBT positive cells.

## 3. Dichlorofluorescein Assay (DCF)

A modified version of the assay reported by Bass et al. (133) was utilized. Glucose (100  $\mu$ l of 50 mM) and 2'7' dichlorofluorescein diacetate (DCF-DA, 50  $\mu$ l of .1 mM) were added to 0.85 ml of cells at  $10^6$ /ml in RPMI 1640. DCF-DA is taken up through the cell membrane and cytosolic esterases deacetylate DCF-DA yielding 2'7' dichlorofluorescein (DCFH). This was incubated at  $37^{\circ}$  C for 10 minutes and appropriate stimulants added. DCFH is rapidly oxidized to 2'7' dichlorofluorescein



(DCF) in the presence of  $H_2O_2$ . DCF is fluorescent and increases in fluorescent intensity are proportional to the amount of  $H_2O_2$  produced. The fluorescence was determined on the Epics V Sorter (Coulter) at time zero and compared to 15 minutes post stimulation. Analysis was performed with an Argon laser set at 300 mW power and 488 nM excitation. Comparison of forward light scatter and  $90^\circ$  light scatter was used to gate PMNs from lymphocytes, monocytes and debris. Green fluorescence from DCF was collected through a 525 nM bandpass filter on a linear scale.

#### G. SIGNAL TRANSDUCTION STUDIES-PERTUSSIS TOXIN

PMNs separated on percol gradients were resuspended at  $10^7$  /ml in RPMI 1640. Pertussis toxin (kindly provided by Dr. Tom Cote) was added at concentrations determined appropriate. Doses in the range of 50 ng/ml to 500 ng/ml were assessed to insure optimal inhibition and determine if the effects are dose related. PMNs were incubated 60 minutes or 120 minutes at  $37^\circ$  C, 5%  $CO_2$  depending on the stimulus. Cells were then washed twice in RPMI 1640, resuspended in RPMI 1640, 1% fcs, cell counts adjusted to  $4 \times 10^6$  /ml and assessed for migration to selected stimulants. The viability of pertussis toxin treated PMNs was greater than 95% as determined by trypan blue exclusion.

#### H. BINDING STUDIES-FORMYLPEPTIDE RECEPTOR

Binding assays were performed to correlate the functional results to expression of formylpeptide receptors. Assessment of FMLP binding was performed using modifications of the method reported by Fletcher et al.

(82). PMNs from appropriate species were resuspended in HBSS w/o at  $10^7$  /ml. One volume of cells was added to one volume HBSS w/o containing concentrations of tritiated FMLP (NEN, N-[phenylalanine-ring-2,6- $^3\text{H}(\text{N})$ ], specific activity 60.0 Ci/mM) ranging in concentrations of 0.5 to 200 nM. Nonspecific binding was determined using 20 uM of unlabeled FMLP. Cells and ligand were incubated 45 minutes on ice. Cells were separated from free ligand by centrifugation for 1 minute at 13,000 X G through 0.2 ml of F-50 (General Electric) silicone oil in 0.4 ml Eppendorf tubes. Tubes were then frozen and cell pellets collected by cutting off the tube tips. These were incubated overnight at room temperature in NCS tissue solubilizer (Amersham) while shaking at less than 1000 rpm. Glacial acetic acid (Baker, 34 ul) and 12 mls of 3a20 scintillation fluid (RPI) were then added to each vial. This was shaken for 1 to 4 hours to allow potential chemiluminescence to dissipate. The samples were then counted on a liquid scintillation counter (Beckman). All samples were done in triplicate. Approximate receptor affinity was determined using Scatchard plots. Initial binding studies utilized filtration through glass filter fiber paper for separation of bound and free ligand. This was found to be an unsuitable assay due to problems with the hydrophobic ligand binding to the paper which led to inconsistent results. Studies using this procedure were therefore discontinued and the centrifugation method described above was employed.

#### I. HPLC ANALYSIS OF FMLP

HPLC was performed on FMLP samples as described by Harvath and Aksamit (134). The FMLP sample (10 ul of 0.1 M) was applied to a



Ultrasphere-ODS  $C_{18}$  column (0.3  $\mu$ M particles, 0.46 X 25 cm) equilibrated in 25% acetonitrile in 0.2 N acetic acid. Compounds were detected by absorbance at 254 nm. The flow rate was 1 ml/min.

#### J. TLC ANALYSIS OF PMA

TLC was performed on PMA samples as previously described (135). PMA (5  $\mu$ l of 0.002 M) was added to precoated silica gel TLC plates (Sigma, 250  $\mu$ M particle size, 60 A pore diameter) with fluorescent indicator. Water saturated diethyl ether was used as the solvent. Samples were visualized with a 254 nm light source.

#### K. DATA REPRESENTATION

Data will be presented for motility assays as either numbers of PMNs migrating per  $\text{mm}^2$ , total numbers of PMNs migrating per well or as stimulation ratios. The stimulation ratio is :

Numbers of PMNs migrating per  $\text{mm}^2$  to FMLP

Numbers of PMNs migrating per  $\text{mm}^2$  randomly.

The stimulation ratio for studies utilizing PMA and A23187 as priming agents is:

Numbers of PMNs migrating per  $\text{mm}^2$  to FMLP post PMA/A23187

Numbers of PMNs migrating per  $\text{mm}^2$  randomly post PMA/A23187

## L. STATISTICS

Statistics were performed with a two tailed students t-test unless otherwise indicated.

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## V. PRELIMINARY FINDINGS

### A. INTRODUCTION

This Chapter will describe results of studies designed as controls or those addressing technical problems encountered during the course of the project.

### B. TECHNICAL STUDIES of DOG PMN MIGRATION

#### 1. Overview

Results from initial studies indicated that the dog PMN migrated quite poorly when compared to other species. Therefore, experiments were undertaken to examine a number of technical parameters related to the migration assay. The goals of this work were to optimize the migration assay for dog PMNs and to confirm that the low percentages of PMNs migrating were not due to technical limitations.

#### 2. Comparison of Techniques to Isolate Dog PMNs

Migration of dog PMNs separated by several techniques was compared to determine the most appropriate means of PMN isolation. Separation techniques included dual percol gradients, dual ficoll gradients, ficoll followed by dextran sedimentation and dextran sedimentation. These procedures were chosen after surveying commonly cited methods in the literature. Migration of PMNs both randomly and to PAF were then

assessed and the separation protocol selected which produced greater than 85% PMNs and optimal migration to PAF. PAF was chosen as a stimulant for this work because preliminary studies indicated that PAF was a potent and reproducible stimulant for dog PMNs, in contrast to low concentrations of FMLP.

Prior to comparing migration of PMNs obtained by each isolation procedure it was important to document the cellular composition of resulting preparations. To compare PMN purity, differential counts were performed on slides of cells obtained from each of the isolation procedures. The results are summarized in Table 2. Dextran sedimentation yielded only 67% PMNs while the other three protocols ranged from 85 to 91% PMNs. No significant differences in migration were noted between PMNs isolated by the four methodologies examined. Dual percol gradients were chosen as the separation procedure for subsequent studies. These findings are similar to those of Grisham et al. where no differences in human PMN migration were found after separation using 4 protocols (136).

### 3. Evaluation of Dog PMN Density and Migration Capacity

The relationship between PMN concentration and numbers of PMNs migrating was examined to confirm that the number of cells migrating was linear in relation to the concentrations of PMNs initially seeded into the chemotactic chamber. When high concentrations of PMNs were utilized for the assay, steric hindrance occurred and the relationship became nonlinear. Performance of migration assays with cell preparations in the nonlinear range leads to inaccuracies in the calculation of



TABLE 2

COMPARISON OF THE EFFECTS OF DIFFERENT  
SEPARATION TECHNIQUES ON DOG PMN PURITY

Separation Means	% PMNs	% Bands	% MNCs
Ficoll-Dextran	88.8 +/- 4.1	7.0 +/- 3.6	4.5 +/- 1.3
Dual Percol	85.5 +/- 4.4	4.2 +/- 1.9	8.0 +/- 2.3
Dual Ficoll	90.8 +/- 7.0	2.0 +/- 1.3	8.0 +/- 5.7
Dextran	67.0 +/- 5.1	3.8 +/- 1.5	30.0 +/- 9.9

All values represent mean +/- SEM

percentages of responsive cells. PMN preparations at varying concentrations were inoculated into the upper compartment of the chemotactic chamber and numbers of cells which migrated randomly and in response to PAF quantitated. There was found to be a linear relationship between cell numbers migrating and numbers of cells originally seeded into the chamber at concentrations ranging from 1 to  $4 \times 10^6$ /ml. The concentration chosen was  $4 \times 10^6$ /ml since the most reproducible results were obtained at this value. This was high compared to other protocols. However, dog PMN migration is sluggish compared to other species and a higher cell concentration is necessary to insure consistent migration.

#### 4. Assessment of Optimal Migration Time for Dog PMNs

The incubation time required for maximal PMN migration was also determined in initial experiments. Human PMNs have been shown to migrate as quickly as 15 minutes and maximally after 30 to 60 minutes (137). Dog PMNs had low random migration as well as a low percentage of cells migrating to stimulants. It was desirable to establish the optimum time necessary to migrate and confirm that fewer cells migrated, as opposed to migration at a slower velocity. Numbers of cells migrating were quantitated after 30, 60 and 120 minutes incubation with either media (random migration) or PAF. Additionally, the numbers of cells which fell off the polycarbonate filter after migration was assessed to confirm that the majority of the cells which migrated remained adherent. Previous work has shown that cells falling off after migration is an important variable to consider in interpreting migration results with this method (137).



Quantitation of numbers of PMNs migrating can be grossly underestimated if large numbers of cells have fallen off the filter. Studies evaluating optimal migration times indicated that dog PMNs migrated as rapidly as 30 minutes, plateaued by 60 minutes and dropped precipitously after 120 minutes incubation. This drop in migrating cell numbers correlated with increases in cell numbers falling from the filter paper into the lower well. Based on these studies, 60 minutes was chosen as the routine incubation time for dog PMN migration.

## 5. Characterization of Dog PMN Stimulants

There were several purposes for these studies. First, it was crucial to identify mediators which were effective stimulants of dog PMNs, particularly since these cells were nonresponsive to FMLP. Additionally, the dog has been a valuable large animal model, crucial in the research of a variety of pathological conditions. Studies defining the normal physiology of these PMNs are conspicuously absent from the literature. This series of studies was directed at evaluation of a variety of mediators as stimulants, characterization of types of motility elicited and the role of G regulatory proteins in these responses.

### a. Identification of Dog PMN Stimulants

Dose response curves for migration to PAF and normal dog sera (NDS) are shown in Figures 1 and 2. Dog PMNs migrated to PAF at concentrations ranging from 0.1 to 10  $\mu$ M with a maximum of  $19.11 \pm 3.28\%$  of cells migrating to 0.1  $\mu$ M PAF. A dilution of

Figure 1. The dose response curve of dog PMN migration to PAF. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 6 animals. \*\* represents migration significantly different from random, ( $P < 0.01$ ) and \*\*\* represents migration significantly different from random ( $P < 0.005$ ).



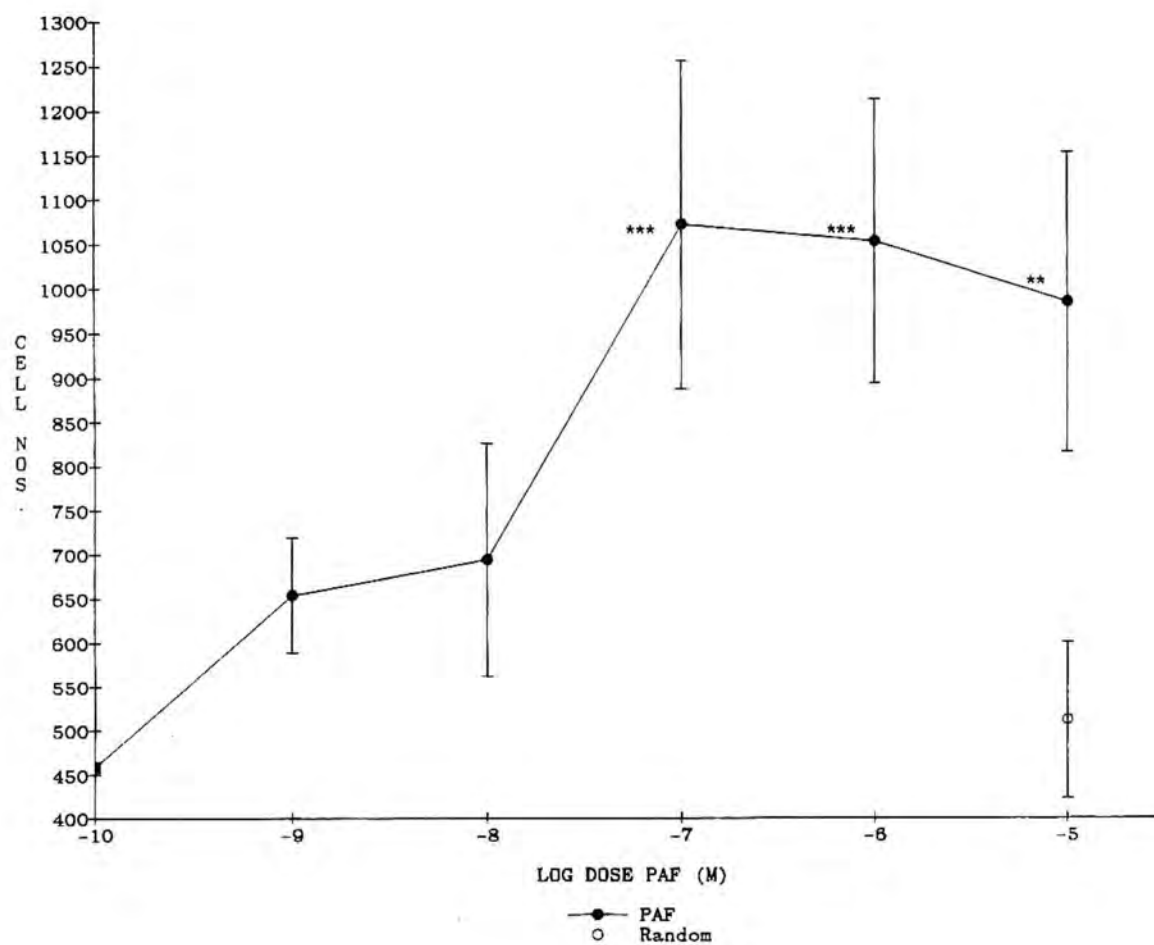
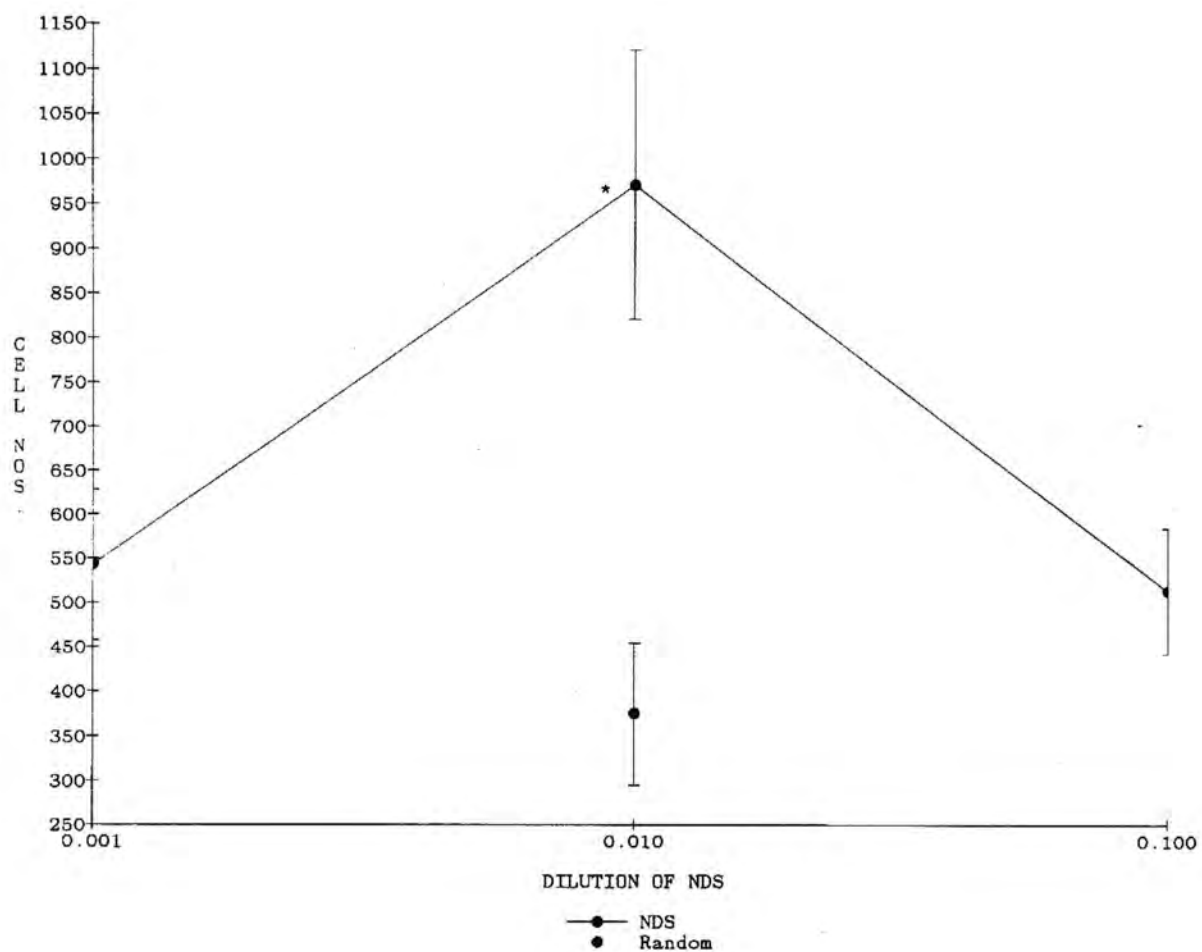


Figure 2. The dose response curve of dog PMN migration to NDS. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 6 animals. \* represents migration significantly different from random, ( $P < 0.05$ ).





1/100 NDS yielded  $17.33 \pm 2.68\%$  cells migrating. Interestingly neither stimulant evaluated produced more than 20% of the cells migrating. Only  $6.90 \pm 1.1\%$  dog PMNs migrated to 0.1  $\mu$ M FMLP. This is in contrast to the 36% of human PMNs migrating to FMLP. Table 3 summarizes dog PMN migration to the stimulants tested.

b. Assessment of the Chemokinetic Component of Dog

PMN Migration

Chemokinesis is an increase in motility due to alterations in speed (orthokinesis) and/ or changes in direction (klinokinesis), while chemotaxis represents an increased migration in response to a gradient of mediators. An important issue in motility studies is discerning chemokinetic and directed (chemotactic) motility. Total migration of human PMNs to FMLP and PAF was significantly greater than chemokinetic migration. However, chemokinesis was 75% and 65% of migration to FMLP and PAF, respectively. The chemokinetic response of dog PMNs to PAF was 66% of migration while chemokinesis in response to NDS was 56%. Total migration was significantly greater than chemokinesis for both stimulants.

c. Signal Transduction Studies for Dog PMNs

To confirm suspected similarities between the mechanism of dog PMN signal transduction and that of human, as well as provide the appropriate controls for subsequent studies using PT, dog PMNs were pretreated with PT and evaluated for migration to PAF and NDS. Prior to this work, experiments were performed with human PMNs, to confirm that results of other laboratories could be reproduced. Pretreatment of human PMNs with 200 ng/ml pertussis toxin for 120

**TABLE 3**  
**MIGRATION RESPONSES OF NORMAL DOG PMNs**

Stimulant	Conc.	Cell Nos.	Ratio
Media (n=11)	--	370 +/- 52	1.0
Normal Dog Serum (n=14)	1%	1111 +/- 108	2.5 +/- 0.19
PAF (n=6)	1.0 uM	915 +/- 189	2.1 +/- 0.65
FMLP (n=6)	0.1 uM	342 +/- 54	1.3 +/- 0.46

All values represent mean +/- SEM and represent numbers of PMNs migrating per mm<sup>2</sup>

minutes inhibited migration to FMLP by 61.2%. The human studies also served as positive controls for the treatment protocol as well as for the pertussis toxin. Pretreatment of dog PMNs with 200 ng/ml PT for 120 minutes completely inhibited migration to both PAF and NDS. Figure 3 illustrates the 74% inhibition of migration to 1% NDS and 84% inhibition in migration to 1 uM PAF.

### C. CHARACTERIZATION STUDIES OF RHESUS MONKEY PMNs

#### 1. Normal Rhesus Monkey PMN Functional Responses

##### a. Migration

Prior to initiation of priming studies, experiments were performed characterizing normal monkey PMN migration. Comparative studies described in the Results Chapter established the poor migration of rhesus monkey PMNs to FMLP (See Table 4, page 85 of the Results Chapter for the data). Results from this section provide the positive controls for rhesus monkey PMN migration. Dose response curves of migration to PAF were generated (Figure 4) and comparisons made of optimal concentration of chemoattractant, maximum number of cells migrating as well as evaluation of the stimulation indices. Low percentages of rhesus monkey PMNs ( $20.0 \pm 1.60\%$ ) migrated to PAF with a stimulation ratio of  $1.60 \pm 0.19$ . Since neither FMLP (See Figure 11, page 84 of the Results Chapter for the data) or PAF (Figure 4) were vigorous stimulants, a crude preparation of C5a was prepared from normal monkey serum (zymosan activated sera, ie, ZAS)



Figure 3. The effects of a 120 minute pretreatment with various concentrations of pertussis toxin on dog PMN migration to 1% NDS or 1  $\mu$ M PAF. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 4 animals.

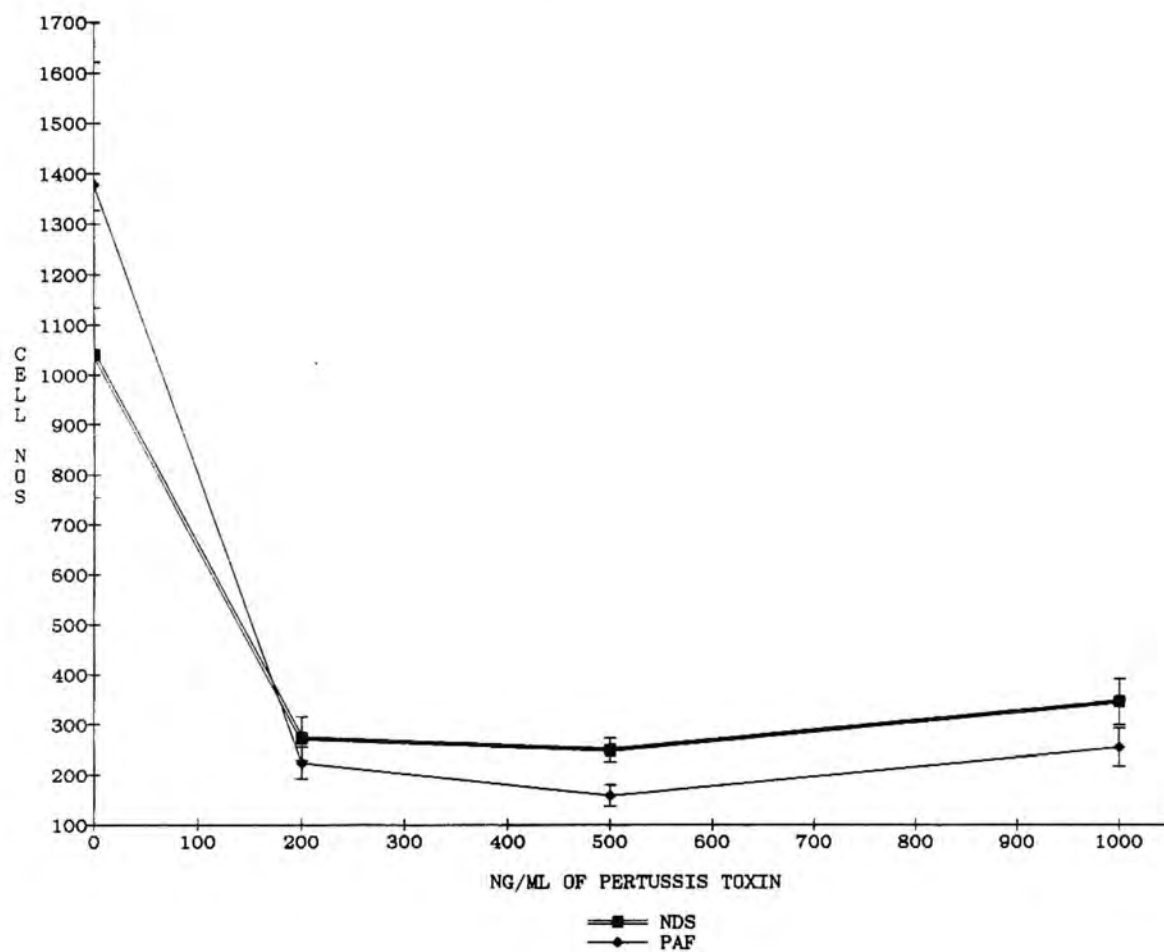
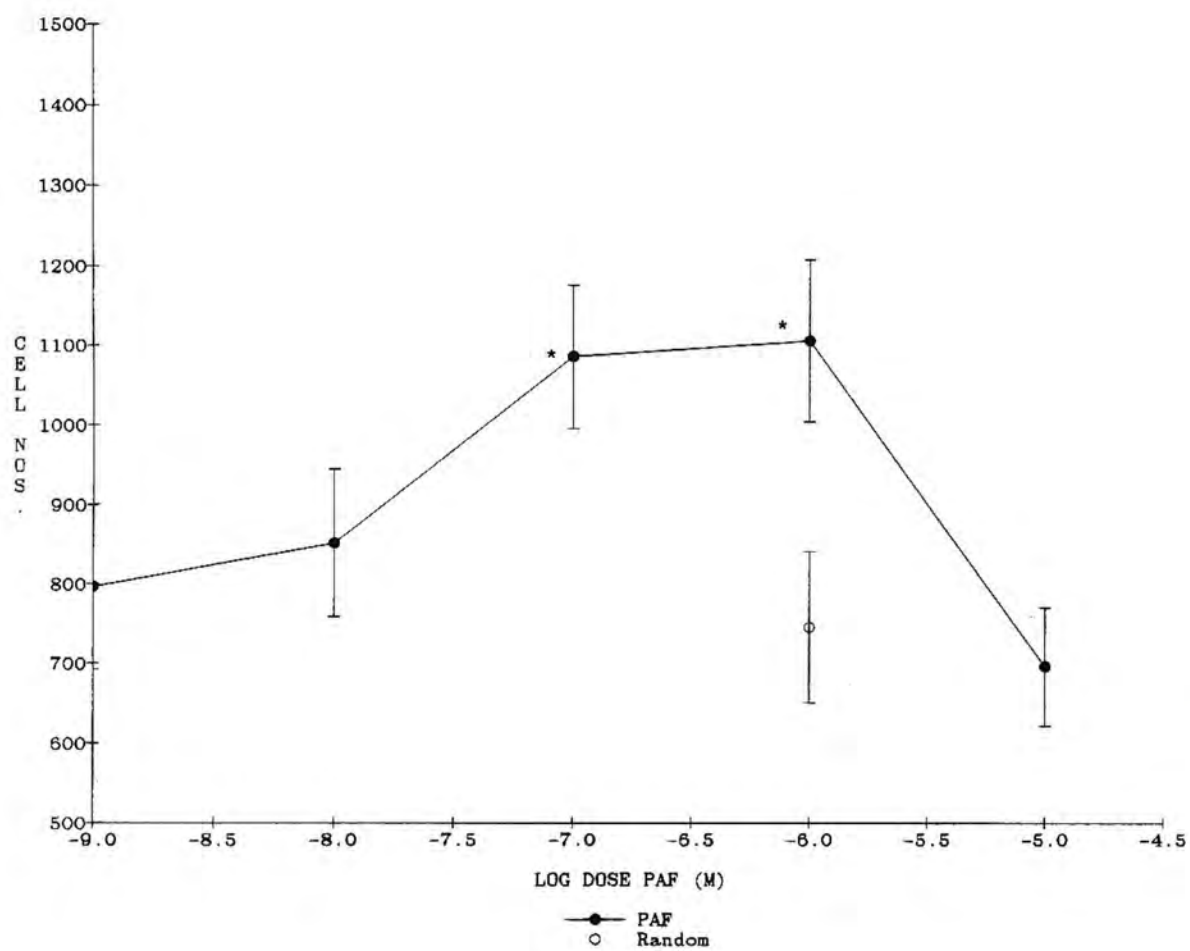


Figure 4. The dose response curve of rhesus monkey PMN migration to PAF. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 21 animals. \* represents migration significantly different from random, ( $P < 0.05$ ).





to be screened as a stimulant. Figure 5 presents the dose response curve for migration of monkey PMNs to ZAS. Rhesus monkey PMN migration to ZAS was brisk, with a stimulation ratio of  $2.70 \pm 0.47$  and  $38.7 \pm 2.4\%$  of the cell migrating.

#### b. Oxidative Burst

Rhesus monkey PMNs were assessed for ability to generate an oxidative burst. The first technique utilized was the NBT reduction assay. Stimulants included FMLP or FMLP in the presence of cytochalasin B. Cytochalasin B enhances responses to receptor mediated stimulation and thus serves as a positive control for these studies. Although the NBT assay is a qualitative measure of PMN oxidative metabolism, the ability to discern percentages of responding PMNs based on assessments of individual cell responses is a major advantage of this assay. Figure 6 shows  $79.5 \pm 4.5\%$  of the rhesus monkey PMNs were NBT positive after 15 minutes exposure to 10  $\mu$ M FMLP and  $96.0 \pm 2.0\%$  were positive in response to 10  $\mu$ M FMLP and 5  $\mu$ g/ml cytochalasin B. The second means utilized to quantitate respiratory burst stimulation was a flow cytometric technique originally described by Bass et al. (133). This procedure monitors relative quantities of  $H_2O_2$  production by PMNs using the dye 2',7'-dichlorofluorescein diacetate (133). This assay was expected to provide percentages of responding versus nonresponding PMNs as well as a relative measure of  $H_2O_2$  production. As a control for subsequent rhesus monkey studies, human PMNs were assayed in preliminary experiments. Studies of the rhesus monkey PMN employed FMLP as a stimulant and PMA was used as a positive control. Preliminary

Figure 5. The dose response curve of rhesus monkey PMN migration to ZAS. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 5 animals. \* represents migration significantly different from random, ( $P < 0.05$ ), \*\*\* represents migration significantly different from random, ( $P < 0.005$ ) and + represents migration significantly different from random, ( $P < 0.0005$ ).



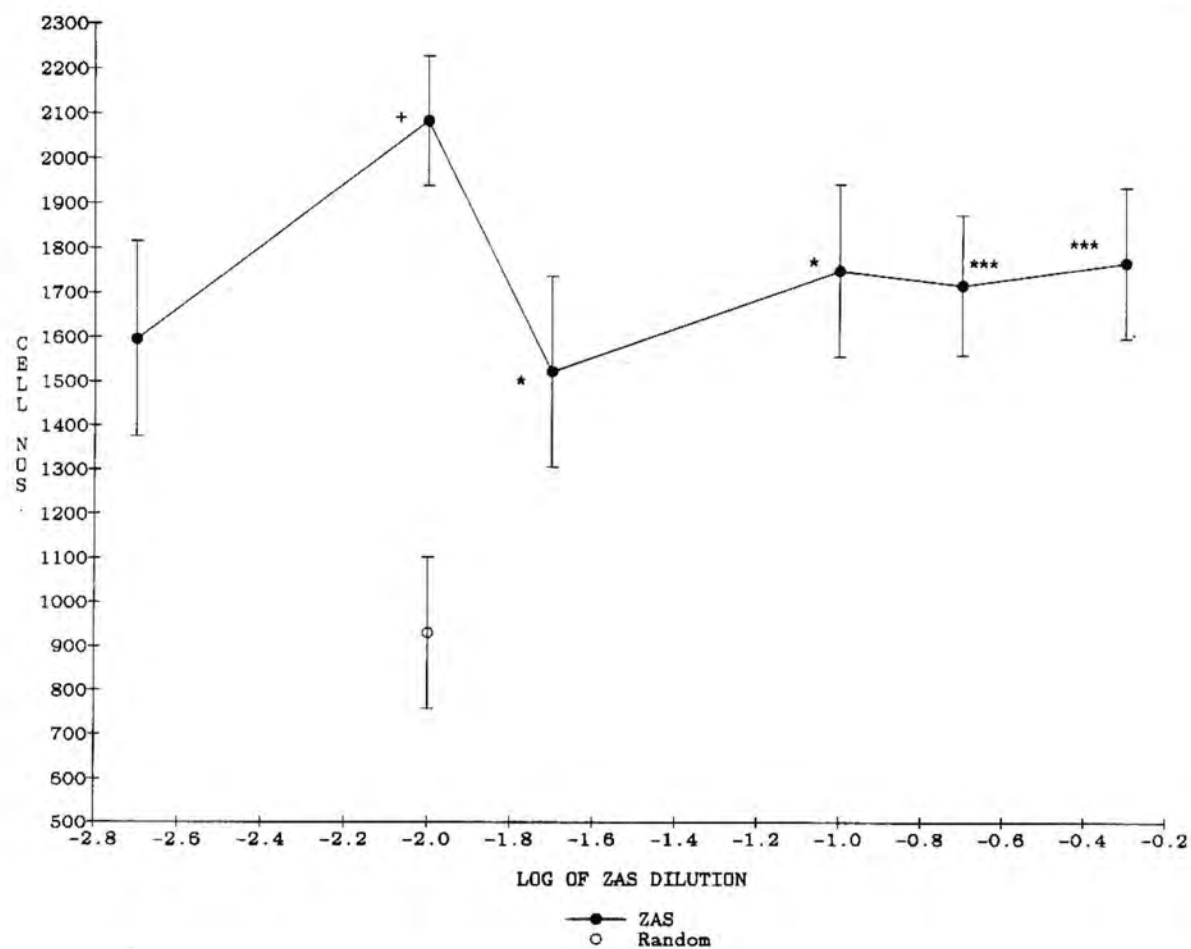
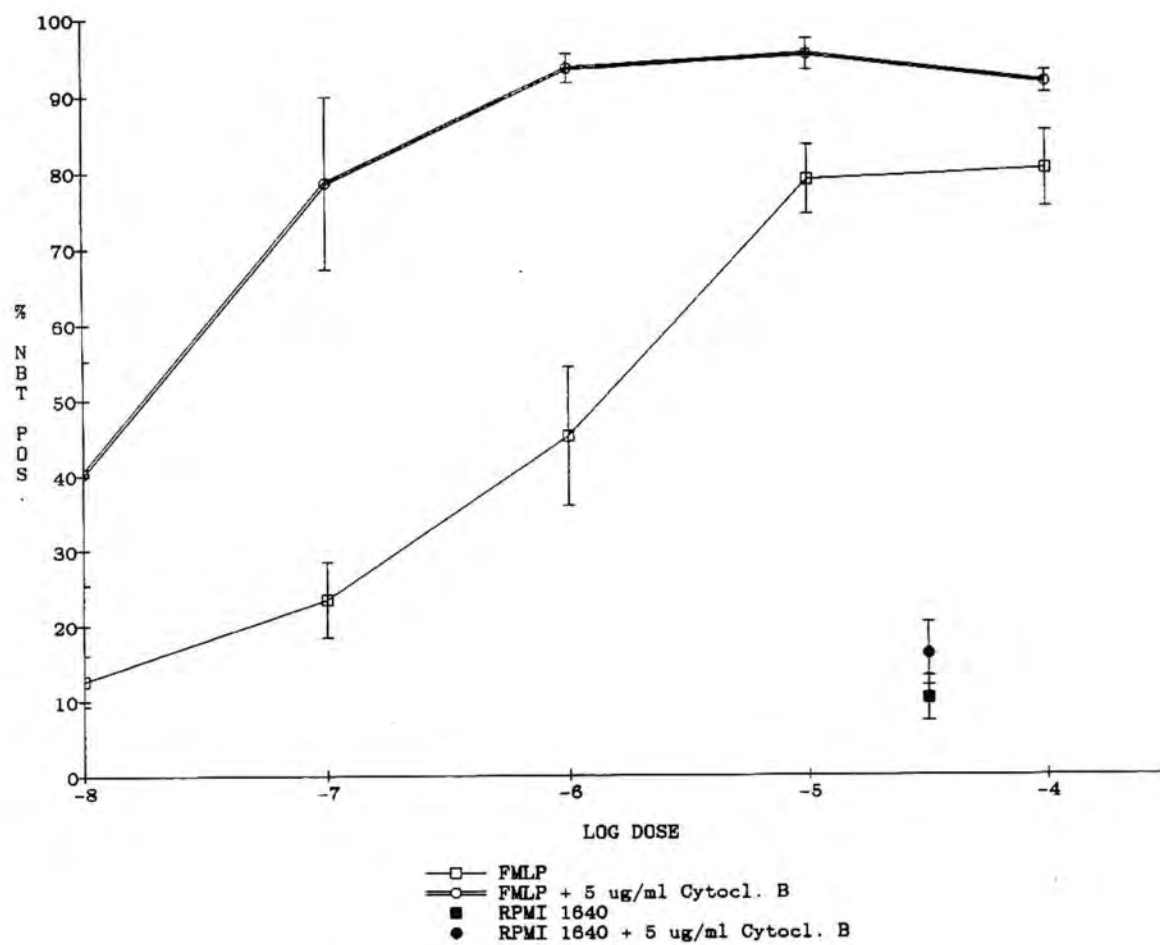


Figure 6. The dose response curve of rhesus monkey PMN NBT reduction in response to FMLP in the presence or absence of 5 ug/ml cytochlasian B. All values are mean  $\pm$  SEM and represent percent NBT positive PMNs. Sample size ranged from 2 to 8 animals.





experiments with human PMNs found 10  $\mu$ M FMLP stimulated a 103% and 118% increase over fluorescence baseline in two human subjects. PMNs from two rhesus monkeys had 104% and 117% increases in fluorescence in response to 10  $\mu$ M FMLP. Results from a representative subject are shown in Figure 7.

One complication of using this technique to measure  $H_2O_2$  production by a stimulant which elicits specific and nonspecific granule release (FMLP) is the dichotomous effects of the myeloperoxidase (MPO) found in nonspecific granules. MPO enhances DCF formation and also degrades  $H_2O_2$  as a result of hypochloride production. The net effect of these actions is a greatly dampened fluorescent signal in comparison to that found with a specific granule secretagogue such as PMA.

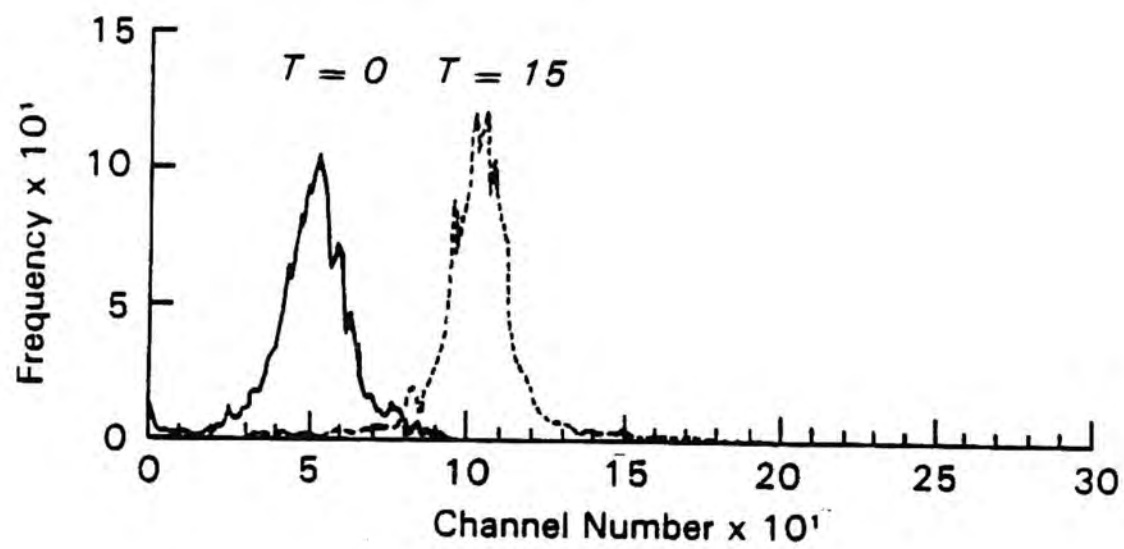
#### D. ANALYTICAL STUDIES

These studies were performed on compounds used during the course of this project for which there was evidence or previous reports of instability.

##### 1. FMLP

Previous work has shown FMLP can be oxidized to a sulfoxide and sulfoxone form (134). Although human monocytes migrate to these oxidized derivatives, human PMNs do not (134). Studies were undertaken to compare the HPLC profiles of frozen aliquots of FMLP prepared at the time of analysis as well as one and three months prior to analysis. The

Figure 7. A representative sample of response to 10  $\mu$ M FMLP by rhesus monkey PMNs in the DCF assay. PMNs were loaded with 2 '7' dichlorofluorescein diacetate (DCF-DA), which is taken up through the membrane. DCF-DA is deacetylated to 2'7' dichlorofluorescein (DCF-H). DCF-H is rapidly oxidized to the fluorescent form, 2'7' dichlorofluorescein (DCF) in the presence of  $H_2O_2$ . Increases in fluorescent intensity are proportional to the amount of  $H_2O_2$  produced. Differences in fluorescence at baseline ( $T=0$ ) versus 15 minutes post FMLP stimulation ( $T=15$ ) were assessed in PMNs preloaded with DCF.



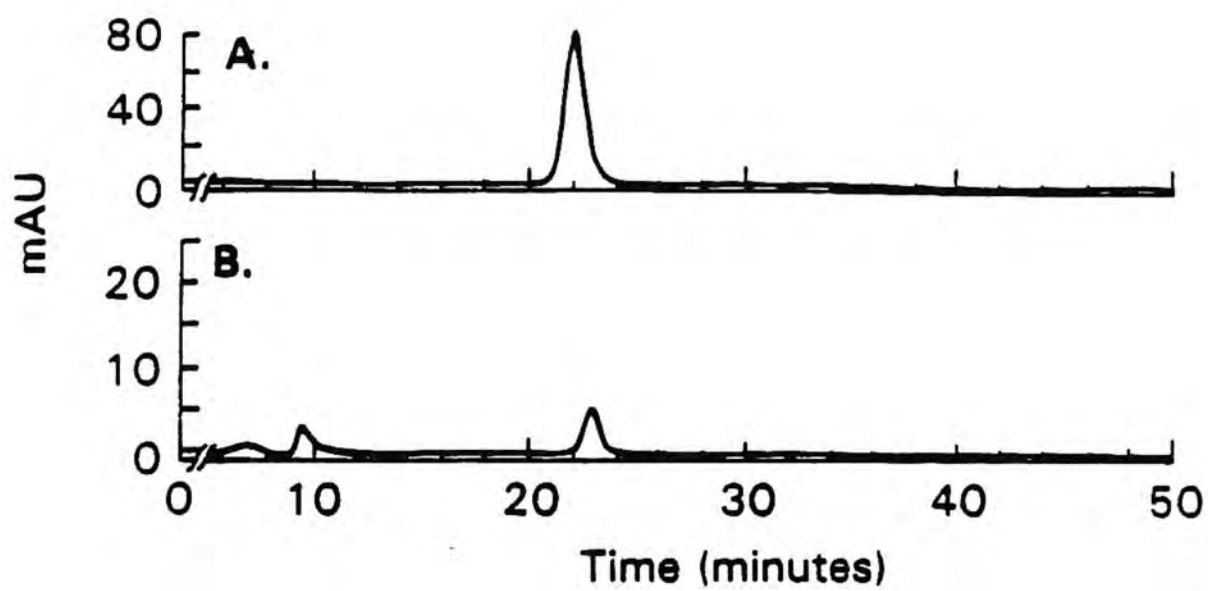


first experiment compared stocks of FMLP freshly prepared and three months old. The fresh preparation had a major peak with a retention time of 22.5 minutes in contrast to the older stock with peaks at 9.7 minutes and 23 minutes (Figure 8). These retention times compared favorably with results of others (134). The second study found no difference in retention times or major peaks of FMLP preparations made fresh or one month old. Based on these studies, FMLP solutions were utilized within one month of preparation.

## 2. PMA

To assure stability of the PMA used for this project, thin layer chromatography (TLC) studies were performed. There were no differences in the  $R_f$  values for the two lots of PMA tested (a  $R_f$  value of 0.35 compared to 0.31). Exposure to light has been reported to cause oxidation of PMA (135). PMA samples were analyzed which had been exposed to light, heat and repeated freeze-thaw cycles. Exposure of samples to light for 96 hours produced a substance having an  $R_f$  value of 0.22, the boiled sample had two compounds with  $R_f$  values of 0.34 and 0.107, while a sample subjected to repeated freeze thaw cycles had an  $R_f$  of 0.33. These studies were a positive control for the ability of the TLC assay to detect metabolites of PMA.

Figure 8. HPLC analysis of a fresh (panel A) and 3 month old batch of FMLP (panel B).





## VI. RESULTS

### A. COMPARATIVE STUDIES OF MIGRATION TO FMLP

#### 1. Overview

The hypothesis of this project predicts that species poorly responsive to FMLP can be converted to a responsive state. Prior to addressing this question it was important to assess the functional responses of PMNs from the species to be studied. Studies were performed to compare FMLP stimulated migration and production of reactive oxygen intermediates by PMNs of dogs, rhesus monkeys and humans. These results will be supplemented by data characterizing expression of the receptor for the chemotactic peptide in these three species. Previous work has shown dog (12, 13) and rhesus monkey PMNs (107, 108) to be poorly responsive or refractory to FMLP while PMNs from humans (3) are vigorous responders. These experiments were conducted to confirm previous work and to serve as baseline studies for the experiments on modulation of FMLP responses.

#### 2. Comparison of Migration to FMLP by Dog, Rhesus Monkey and Human PMNs

Comparisons were made among the dose response curves for the migration to FMLP by PMNs from dogs, rhesus monkeys and humans. PMNs isolated from the peripheral blood of normal dogs were evaluated for migration to concentrations of FMLP ranging from 10 fM to 1 uM. The

data in Figure 9 indicate that the migration of dog PMNs to FMLP was not significantly different from random at any concentration evaluated. Migration of human PMNs was next evaluated as a positive control for both the FMLP preparation and the assay conditions. Figure 10 illustrates the dose response curve of human PMN migration to FMLP. The FMLP concentration stimulating maximum migration was 0.1  $\mu$ M, eliciting  $35.8 \pm 9.0\%$  of the PMNs to migrate. In contrast to the dog, a significantly greater number of rhesus monkey PMNs migrated to FMLP when compared to numbers migrating randomly (Figure 11). A 0.1  $\mu$ M concentration of FMLP elicited a stimulation ratio of  $1.52 \pm 0.13$  from rhesus monkey PMNs which was significantly less than the  $3.88 \pm 0.40$  of human PMNs (Table 4,  $P < 0.0001$ ). This concentration of FMLP (0.1  $\mu$ M) also stimulated  $19.35 \pm 1.76\%$  of the rhesus monkey PMNs to migrate. Table 4 summarizes a comparison of FMLP induced migration in human, dog and rhesus monkey PMNs. This work confirms previous observations that human PMNs migrate vigorously to FMLP and establish that in this assay system the monkey response is 50% of that seen in humans. The differences between the dose response curves of the rhesus monkey PMN and that of the human were noteworthy (Figures 10 and 11). Striking differences were observed in concentrations of stimulant necessary for maximum migration, as well as desensitization. The concentration of FMLP stimulating maximum migration in rhesus monkeys was 100  $\mu$ M as compared to 0.1  $\mu$ M in humans. Human PMN migration decreased at concentrations of FMLP greater than 1  $\mu$ M (Figure 10) while monkey PMN migration remained close to maximum from 100  $\mu$ M to 0.1  $\mu$ M (Figure 11). These data suggest differences in the chemotactic deactivation process of these two species. Thus, in comparison to human PMNs, the monkey

Figure 9. The dose response curve of dog PMN migration to FMLP. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 6 animals.



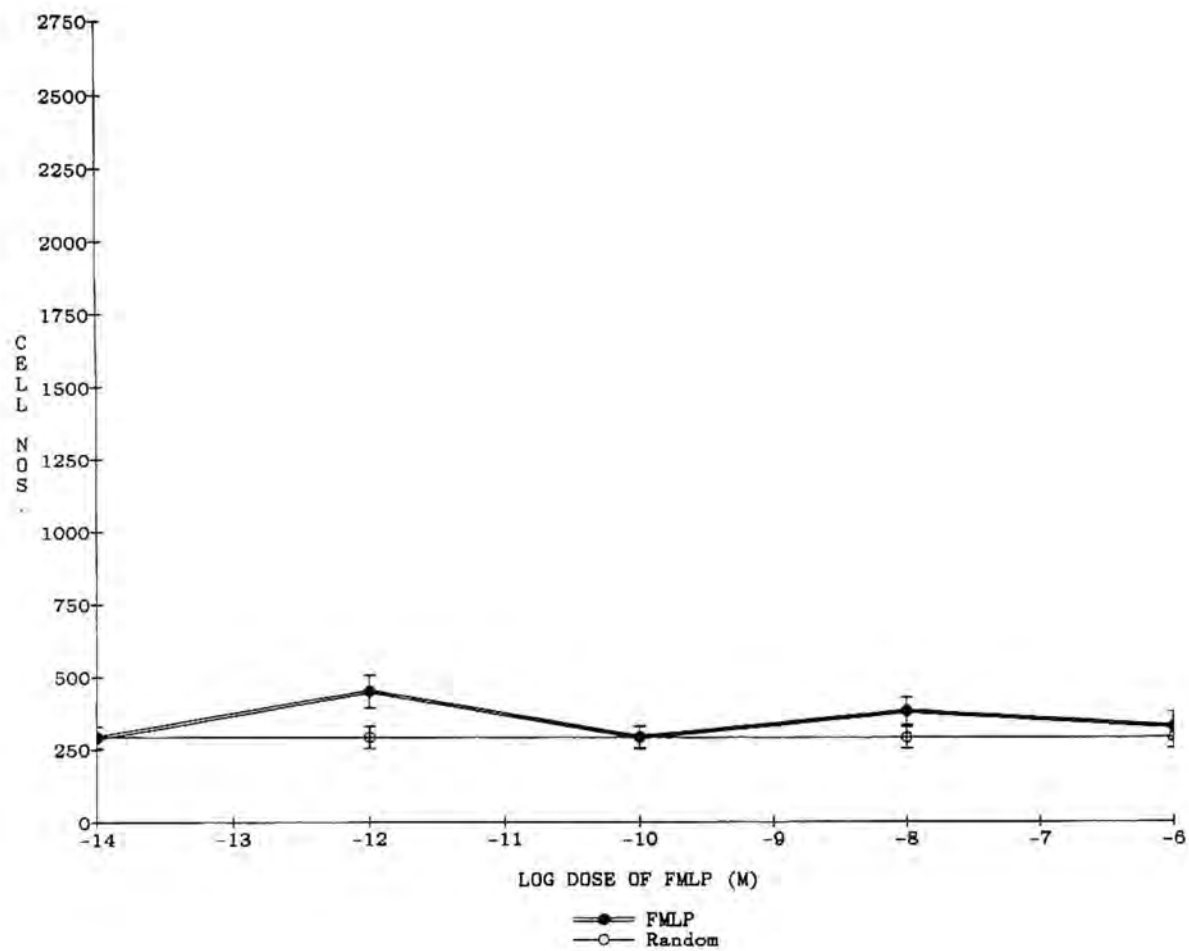


Figure 10. The dose response curve of human PMN migration to FMLP. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 4 subjects. \* represents migration significantly different from random, ( $P < 0.05$ ).

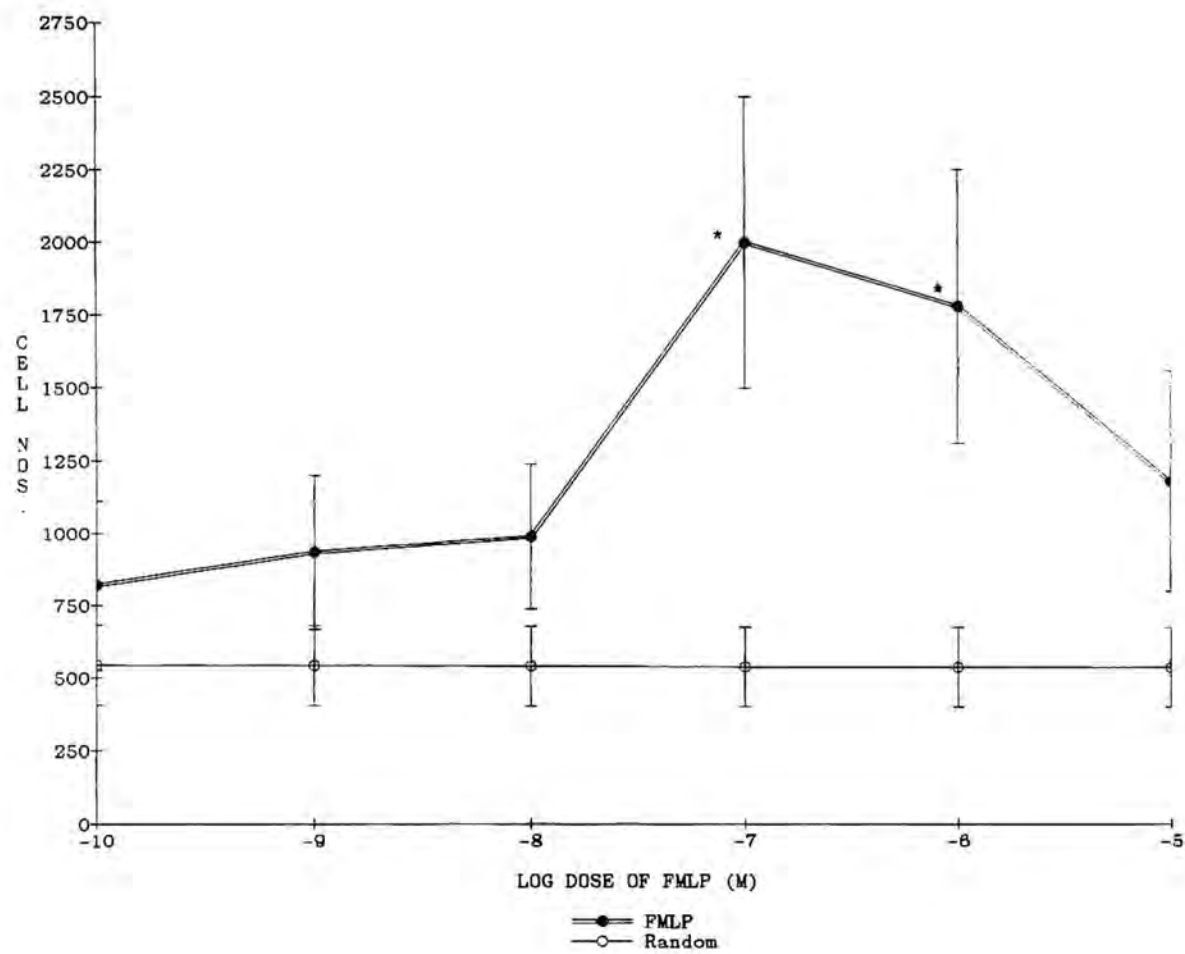




Figure 11. The dose response curve of rhesus monkey PMN migration to FMLP. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 21 animals. \* represents migration significantly different from random, ( $P < 0.05$ ).

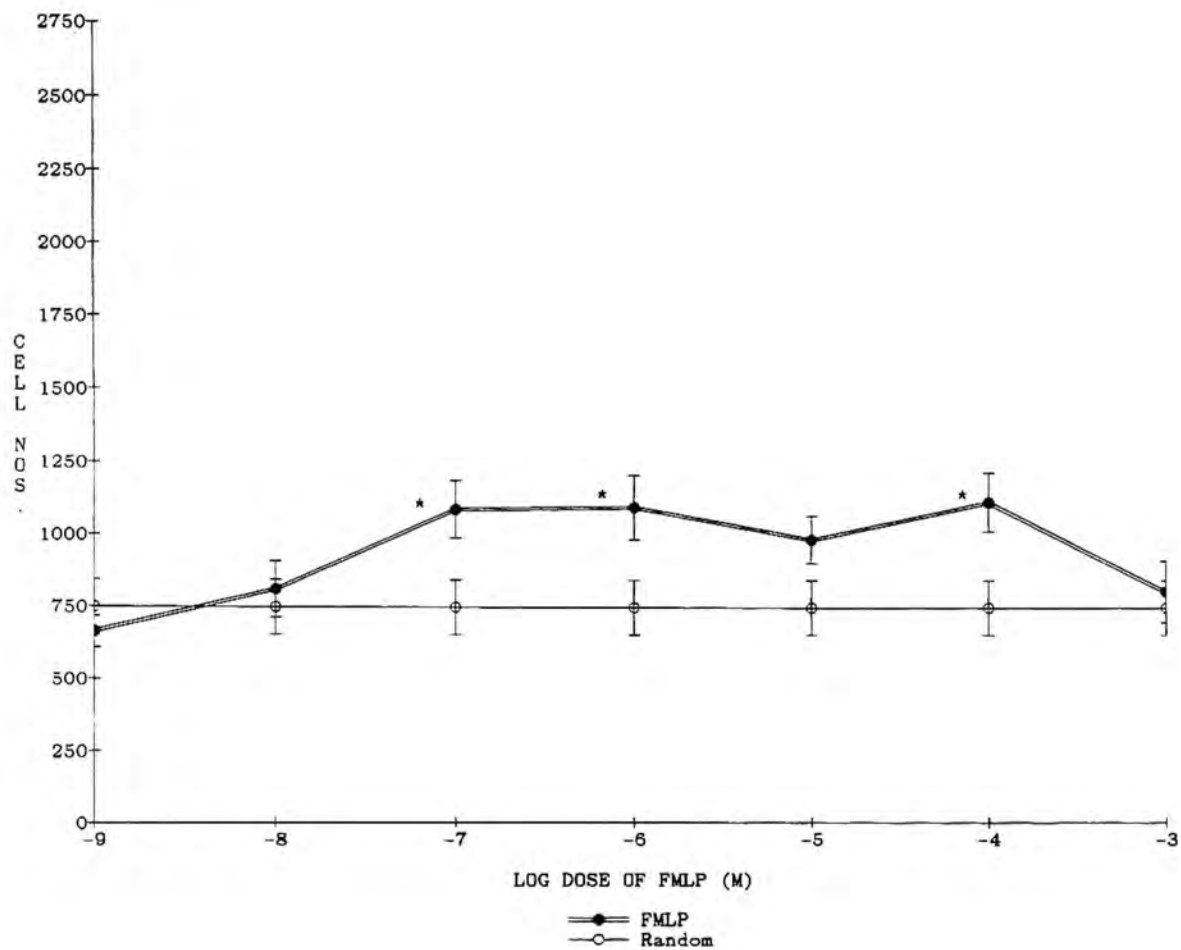


TABLE 4

MIGRATION RESPONSES TO 0.1  $\mu$ M FMLP BY HUMAN,  
RHESUS MONKEY AND DOG PMNs

	Random	FMLP (0.1 $\mu$ M)	Ratio
Human	543 $\pm$ 138	2014 $\pm$ 504	3.88 $\pm$ 0.40
Monkey	748 $\pm$ 95	1088 $\pm$ 99	1.52 $\pm$ 0.13
Dog	293 $\pm$ 38	342 $\pm$ 54	1.29 $\pm$ 0.46

All values represent mean  $\pm$  SEM and represent numbers of  
PMNs migrating per mm<sup>2</sup>

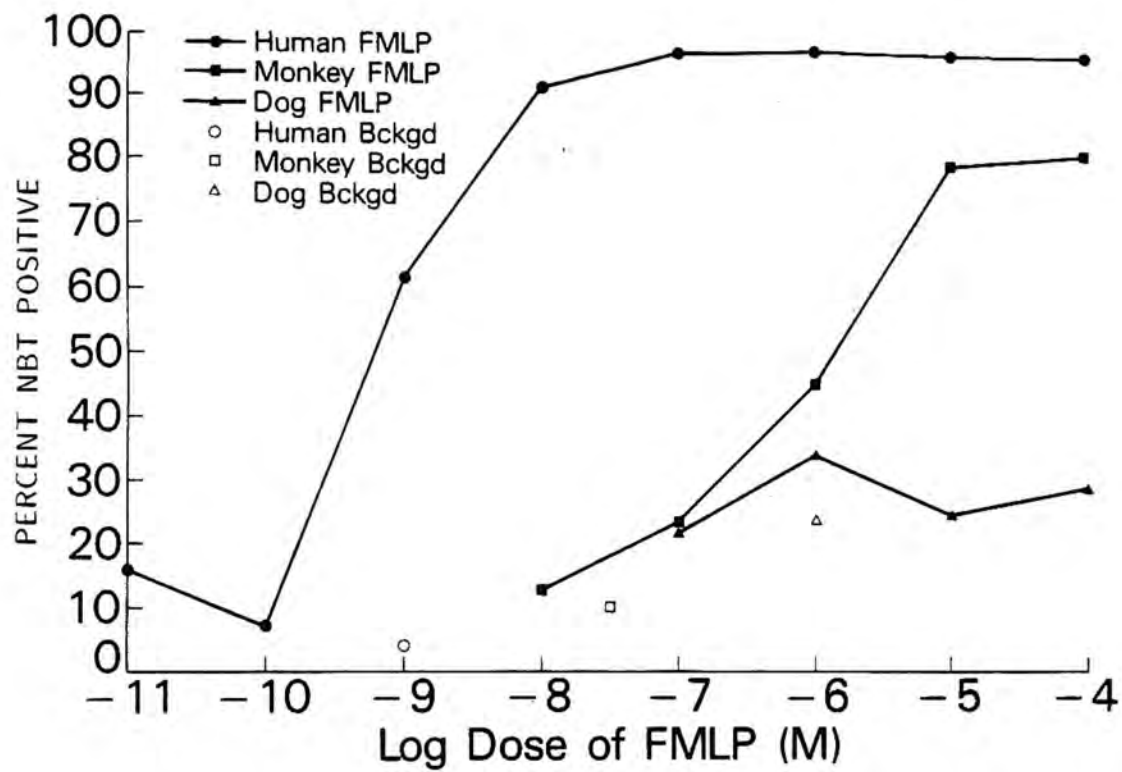


PMN is a poor responder to FMLP, while the dog PMN is virtually nonresponsive.

### 3. Comparative Studies of Oxidative Burst Responses to FMLP

Figure 12 illustrates the difference in the FMLP dose response curves for oxidative metabolism of PMNs from humans, dogs and rhesus monkeys in the NBT assay. PMNs from these three species were treated 15 minutes at 37° C with the appropriate concentration of FMLP in the presence of NBT. Over 90% of human PMNs were NBT positive at FMLP concentrations of 10 nM or greater while 10 uM FMLP was required to produce a maximum of 79.5 +/- 4.5% NBT positive rhesus monkey PMNs. The dog NBT response was the same as media controls at concentrations of 10 uM FMLP or less. Also evident from inspection of Figure 12 are differences in maximum percentages of FMLP responsive PMNs as well as the shape of the FMLP dose response curve for each species. The leftward shift of the human FMLP dose response as compared to that of the rhesus monkey demonstrates that human PMNs are more responsive to the chemotactic peptide than PMNs of the rhesus monkey. Pretreatment with cytochalasin B resulted in a shift to the left of the dose response curve for rhesus monkey PMNs (See Figure 6, page 70 of the Preliminary Findings Chapter for the data). Figure 6 also demonstrates that virtually 100% of rhesus monkey PMNs have the capacity to generate an oxidative burst to FMLP. Dog PMNs stimulated with 5 nM PMA were 98% NBT positive PMA indicating these cells were capable of generating a respiratory burst, yet were nonresponsive to FMLP concentrations of 10 nM to 10 uM.

Figure 12. The dose response curve of NBT reduction to FMLP by PMNs from humans, rhesus monkeys and dogs. All values are expressed as means and represent percentages of NBT positive PMNs.





#### 4. Formylpeptide Receptor Expression of Dog, Human and Rhesus Monkey PMNs

Studies evaluating FMLP binding sites on PMNs from dogs, humans and rhesus monkeys were performed. Estimations of the affinities for the formylpeptide receptors using Scatchard analysis from 2 human samples were 2.8 and 44 nM for 1 subject and 2.2 and 62 nM for the other. These values are in the range reported by other laboratories for human formylpeptide receptors (8). Binding studies conducted with rhesus monkey PMNs in identical experimental conditions as human PMNs resulted in  $9.1 \pm 0.60\%$  specific binding of FMLP (data not shown). These data along with the presence of functional responses support the existence of formylpeptide receptors on the rhesus monkey, although in low numbers or low affinity. Dog PMNs had no specific binding of FMLP (data not shown).

### B. DOG STUDIES

#### 1. Modulation of Dog PMN Migration to FMLP

##### a. Effects of PMA Pretreatment on Migration of Dog PMNs to FMLP

PMNs pretreated 5 minutes with PMA (0.1 to 10 nM) were assessed for migration to FMLP at concentrations of 1 pM to 1 uM to determine if PMA could enhance migration to FMLP. Due to the myriad of responses PMA stimulates in PMNs, preliminary studies were performed to assess the effects of this compound on random migration (migration in the absence of FMLP). PMA did not induce

statistically significant increases in random migration although there was a variation in responses of individual dogs as Figure 13 A-C illustrates. Based on these observations, migration data for the FMLP studies will be presented as stimulation ratios (see Materials and Methods Chapter, Section K for description) so increases in random migration resulting from PMA are removed and the value reflects only increases in migration to FMLP. Pretreatment of PMNs with 1 and 10 nM PMA resulted in enhanced FMLP migration, while 0.1 nM PMA had no effect (Figure 14 A-C). Figure 14 B demonstrates the increased migration to FMLP after 1 nM PMA pretreatment. Statistical differences in migration of PMA treated and control PMNs were noted at 1 pM, 10 nM and 1 uM FMLP ( $P < 0.005$ , 0.006, 0.013 respectively). Statistically significant increases in migration to 0.1 nM FMLP were found after exposure to 10 nM PMA ( $P < 0.01$ ) (Figure 14 C). Figure 15 shows the individual responses of PMNs from 14 dogs to 10 nM FMLP after a 5 minute pretreatment with 1 nM PMA. Stimulation ratios of 11 of the 14 animals tested increased.

Experiments were then performed to assess if migration increases correlated to increased FMLP binding sites. Figure 16 demonstrates the results of studies comparing specific binding of 100 nM  $^3\text{H}$ -FMLP by dog PMNs pretreated with 1 nM PMA for 30 minutes to control PMNs. Although the differences were not significant ( $P < 0.07$ ) there was a trend towards an increase in specific binding of FMLP after the PMA treatment. Normal dog PMNs had  $-0.15 \pm 7.7\%$  specific binding while the PMA treated group bound  $21.7 \pm 7.4\%$  of the labeled FMLP specifically.

Figure 13. The effects of pretreatment of dog PMNs with PMA for 5 minutes on random migration. Each line represents total numbers of PMNs migrated per well in the presence and absence of PMA for each of the animals tested. Panel A represents 0.1 nM PMA pretreatment (n=14), panel B represents 1 nM PMA pretreatment (n=15) and panel C represents 10 nM PMA pretreatment (n=15).



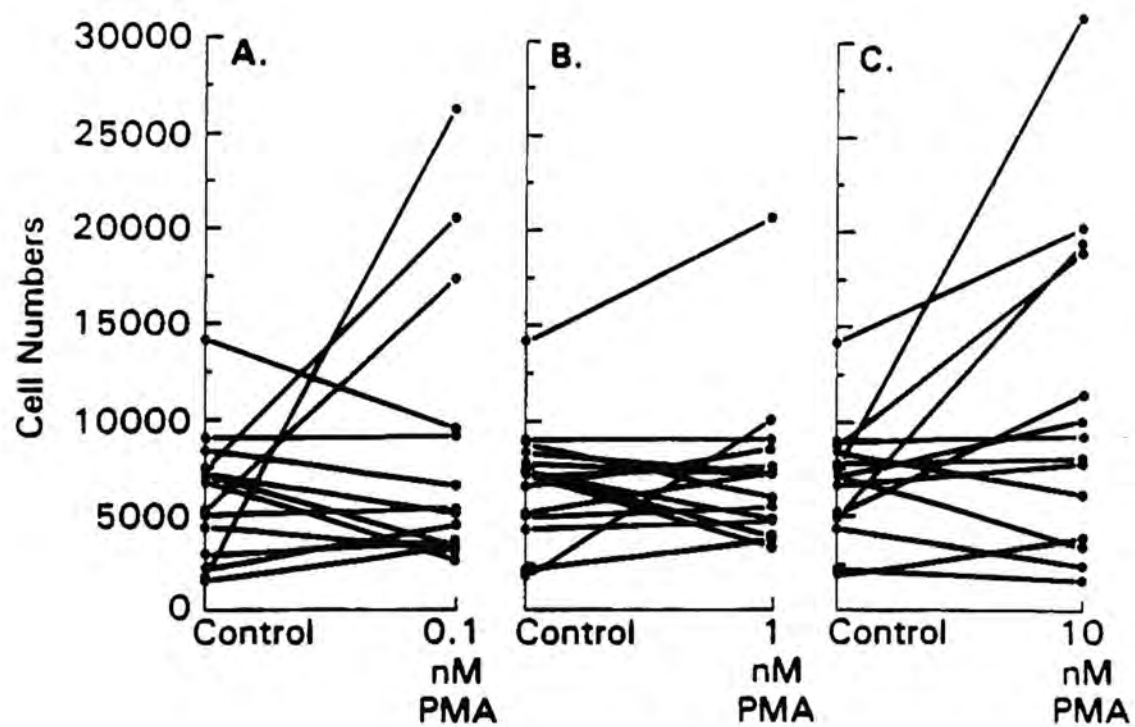


Figure 14. The effects of pretreatment of dog PMNs with PMA for 5 minutes ( ●—● ) on the migration to FMLP (1 pM to 1 uM) compared to responses of control PMNs pretreated for 5 minutes with media ( ▲--▲ ). All values are mean  $\pm$  SEM and represent the stimulation ratio (numbers of PMNs migrating to FMLP after PMA pretreatment divided by numbers of PMNs migrating to media controls after PMA pretreatment). Panel A represents 0.1 nM PMA pretreatment, panel B represents 1 nM PMA and panel C represents 10 nM PMA pretreatment. \* represents a stimulation ratio for PMA treated PMNs significantly different from untreated control, ( $P < 0.05$ ), \*\* represents a stimulation ratio of PMA treated PMNs significantly different from untreated control, ( $P < 0.01$ ) and \*\*\* represents a stimulation ratio for PMA treated PMNs significantly different from untreated control, ( $P < 0.005$ ). The sample size was between 11 and 15 animals.

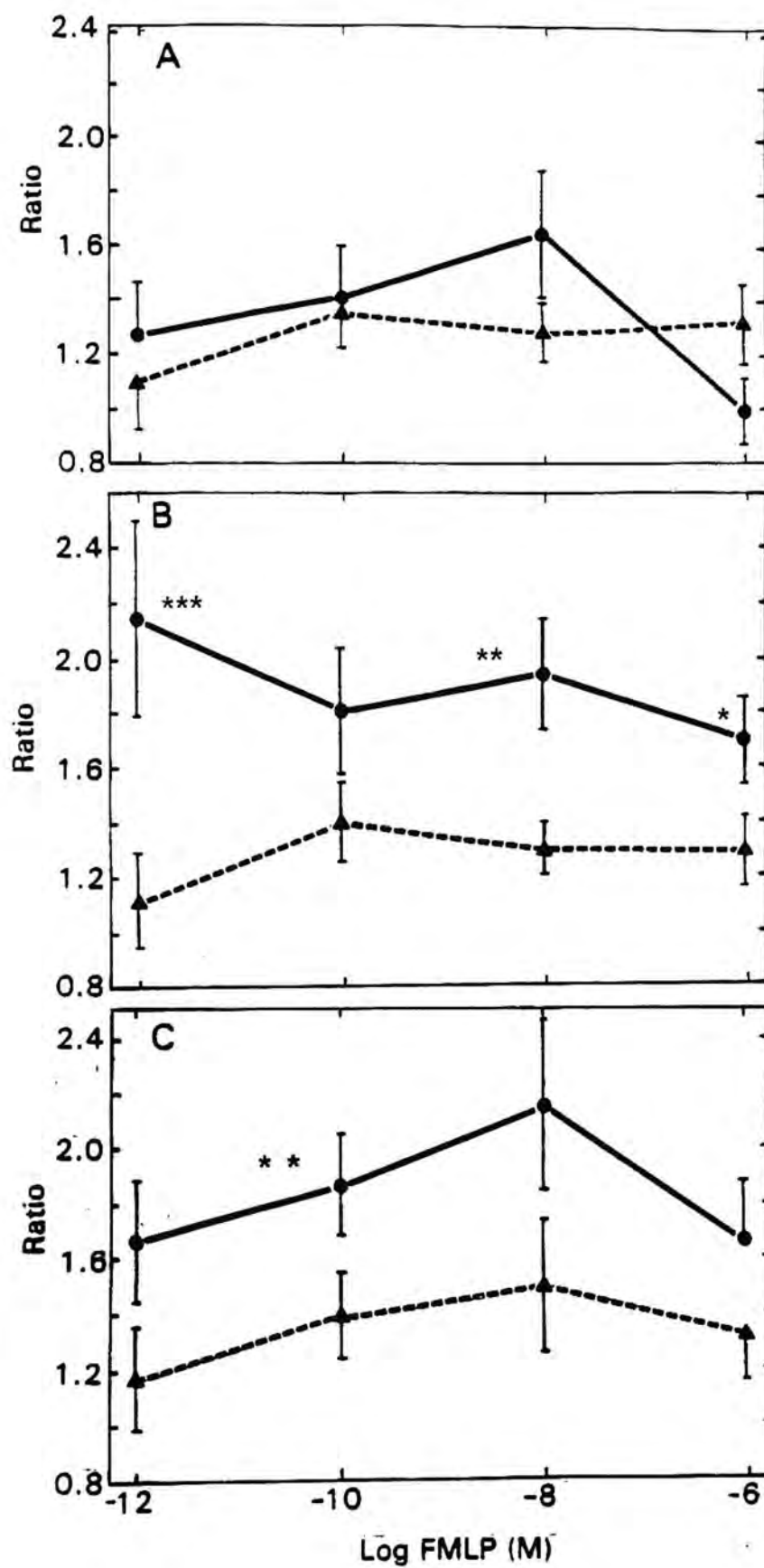




Figure 15. The effects of a 5 minute pretreatment of dog PMNs with 1 nM PMA on migration to 10 nM FMLP. Each line represents the stimulation ratio of PMNs which have migrated to 10 nM FMLP in the presence and absence of PMA for each of the 14 animals tested.

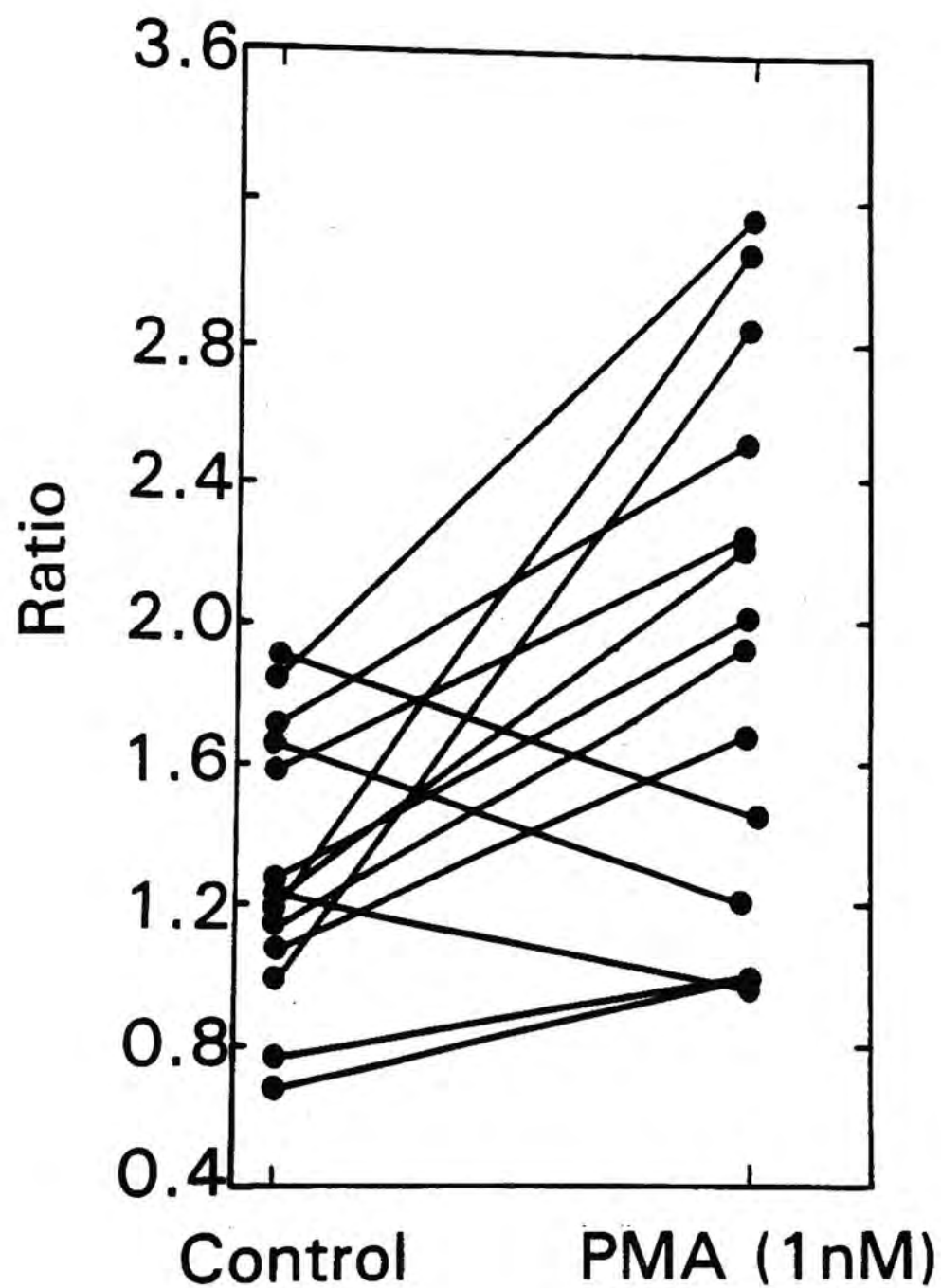
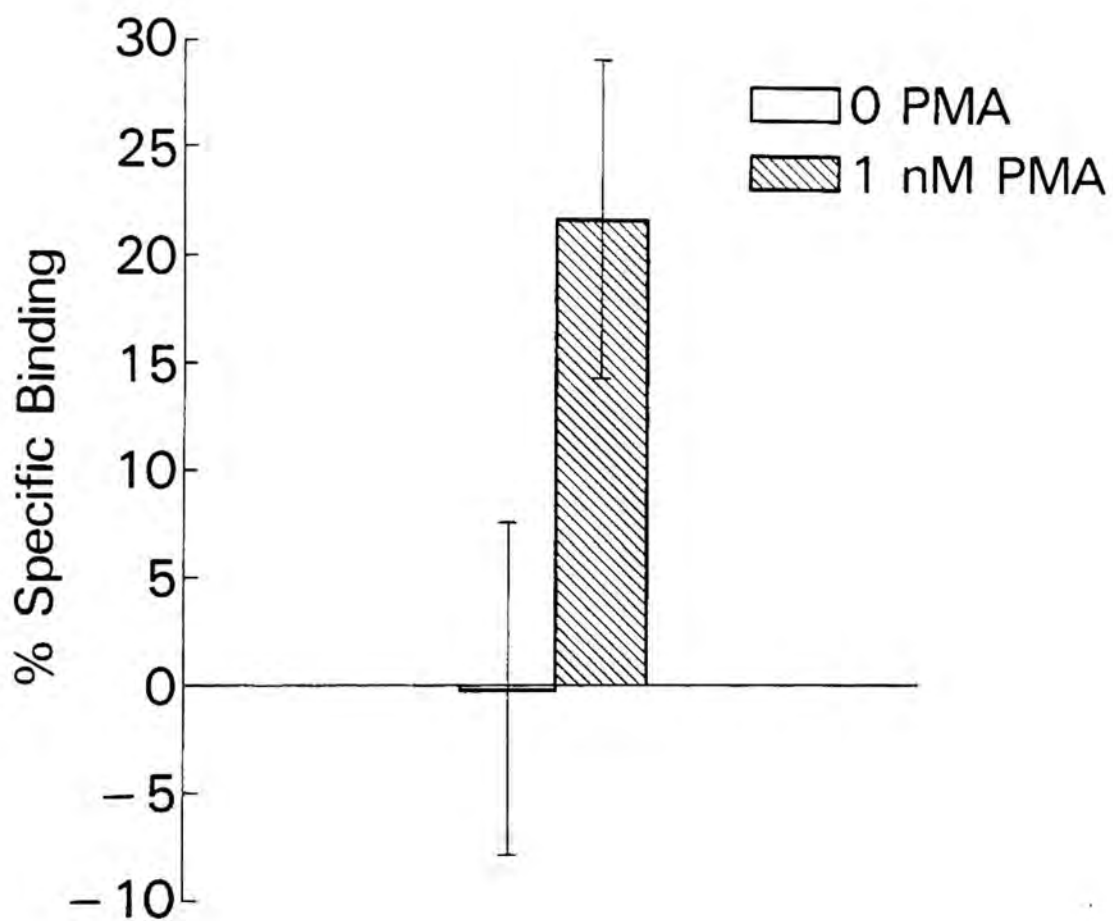


Figure 16. The effects of a 30 minute pretreatment of dog PMNs with 1 nM PMA on specific binding of 100 nM  $^3\text{H}$ -FMLP. All values are percent specific binding and represent mean  $\pm$  SEM. Sample size for PMA treated values was 7 animals and control sample size was 4.





b. Effects of A23187 Pretreatment on Migration of Dog PMNs to FMLP

PMNs from dogs were treated with the calcium ionophore A23187 for 30 minutes, then assessed for migration to FMLP. The purpose of these studies was to determine if other agents shown to mobilize intracellular pools of formylpeptide receptors in human PMNS could induce FMLP migration by dog PMNs. Figure 17 A-C illustrates the results of those experiments with 1, 10 and 100 nM of A23187. No significant differences were found in these studies. There was a suggestion of a trend toward motility increases after pretreatment with 100 nM A23187 in response to 10 nM FMLP ( $P < 0.11$ ), (Figure 17 C). However, of the 14 dogs tested, only 6 had increased responses (Figure 18).

c. n-Butanol Effects on Formylpeptide Receptor Expression by Dog PMNs.

The aliphatic alcohol n-butanol has been shown to increase binding of FMLP as a result of expression of cryptic stores of receptors (61, 62). Preliminary binding studies were performed on PMNs pretreated for 15 minutes at room temperature with 2.5% or 0.25% n-butanol. No significant differences were observed so these studies were not pursued further (data not shown.)

2. Migration of Dog PMNs to E. coli Filtrates

Previous work indicated that E. coli filtrates were stimulants of dog PMN migration (12, 113). Experiments were initiated to confirm these results and further characterize the role of the formylpeptide receptor in

Figure 17. The effects of pretreatment of dog PMNs with A23187 for 30 minutes ( ▲-▲ ) on the migration to FMLP (1 pM to 1 uM) as compared to migration of PMNs treated for 30' with media ( ●-● ). All values are mean  $\pm$  SEM and represent the stimulation ratio (numbers of PMNs migrating to FMLP after A23187 pretreatment divided by numbers of PMNs migrating to media controls after A23187 pretreatment). Panel A represents 1 nM A23187 pretreatment, panel B represents 10 nM A23187 pretreatment and panel C represents 100 nM A23187 pretreatment. Sample size was 14 animals.



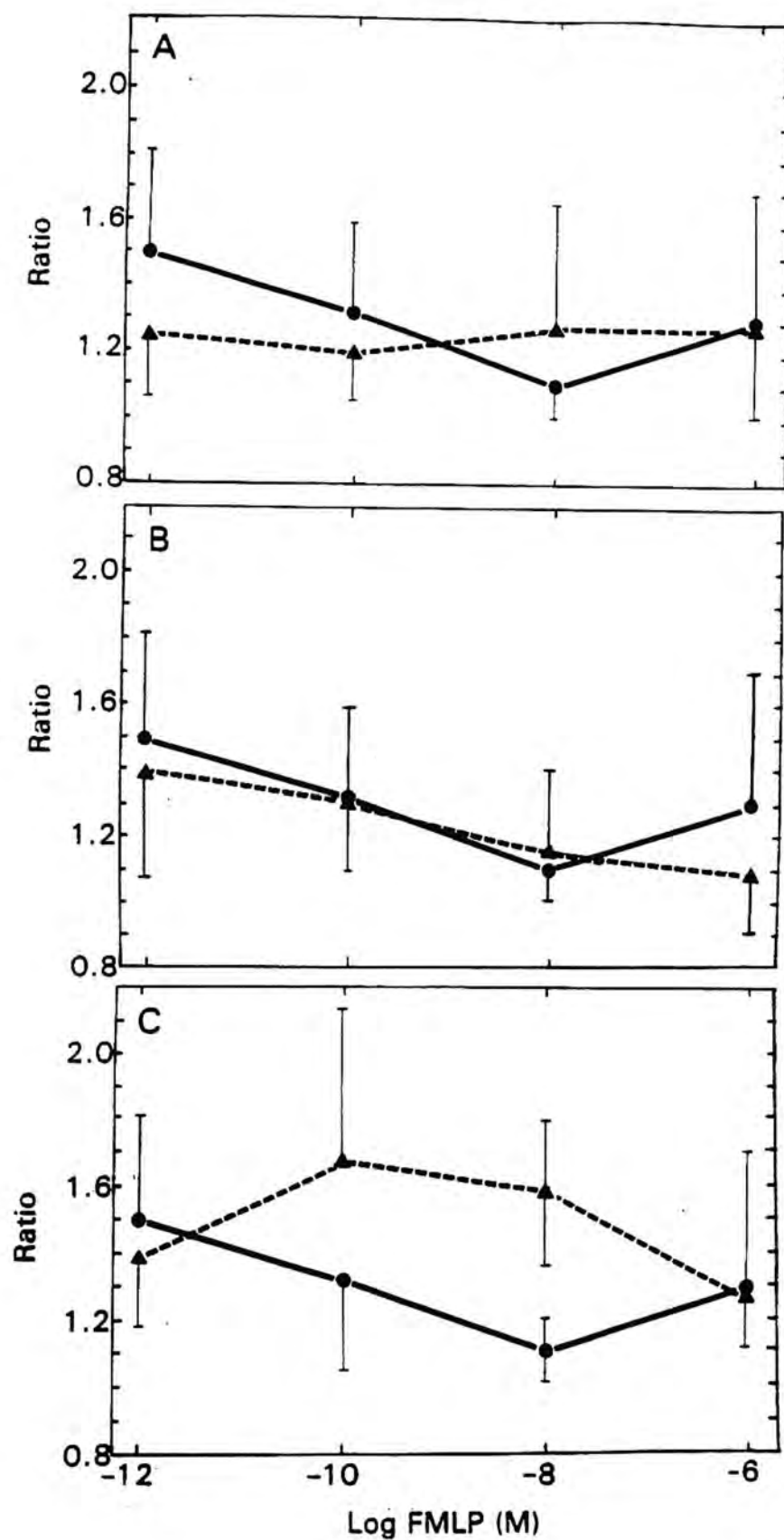
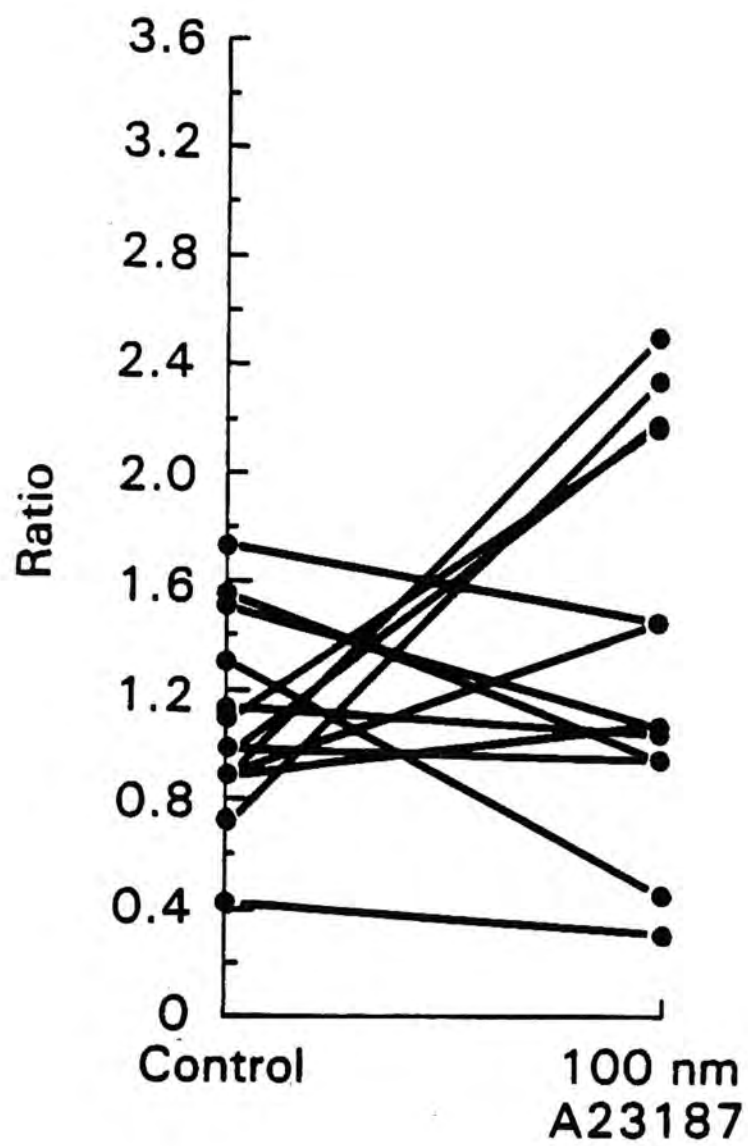


Figure 18. The effects of a 30 minute pretreatment of dog PMNs with 100 nM A23187 on migration to 10 nM FMLP. Each line represents the stimulation ratio of PMNs which have migrated to 10 nM FMLP in the presence and absence of A23187 treatment for each of the 14 animals tested.





those responses. Filtrates from log phase growth E. coli were first tested as chemotactic agents for human PMNs as a positive control. This preparation was a potent stimulant of migration at an optimum dilution of 1/50 (Figure 19). At this dilution of filtrate  $33.07 \pm 3.77\%$  of human PMNs migrated. Dog PMNs also migrated vigorously to the filtrate,  $23.6 \pm 3.08\%$  responding with a stimulation ratio of  $3.05 \pm 0.41$  (Figure 20). Thus, E. coli filtrates proved a more potent stimulant for dog PMNs than either NDS or PAF. Interestingly, the dose response curve was quite different from that for humans. Undiluted filtrates were the only concentration which stimulated dog PMN migration significantly above that of random. This is in contrast to the 1/50 dilution of filtrate optimum for human PMNs. Pretreatment with 200 ng/ml of PT for 120 minutes inhibited migration of dog PMNs to the filtrates (Figure 21). An inhibition of 67.2% was found in comparing PT treated PMNs to those untreated. PT effectively uncouples the chemotactic receptor from phospholipase C. A PT mediated inhibition in migration to E. coli filtrate strongly suggested a receptor coupled migration response. To confirm that migration to the filtrate was elicited through the formylpeptide receptor, studies with the formylpeptide receptor antagonist t-boc-MLP were performed. Control studies established that migration of human PMNs to FMLP was inhibited 58% in the presence of 100 uM t-boc-MLP (Figure 22). A 43.8% inhibition of dog PMN migration to E. coli filtrates was found after pretreatment for 10 minutes with 100 uM of the antagonist (Figure 23). The difference between dog PMN migration to E. coli filtrate in the presence and absence of antagonist was highly significant ( $P < 0.0001$ ), while random migration as well as that to PAF and NDS were not

Figure 19. The dose response curve of human PMN migration to E. coli filtrates. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size ranged from 7 to 15 subjects.

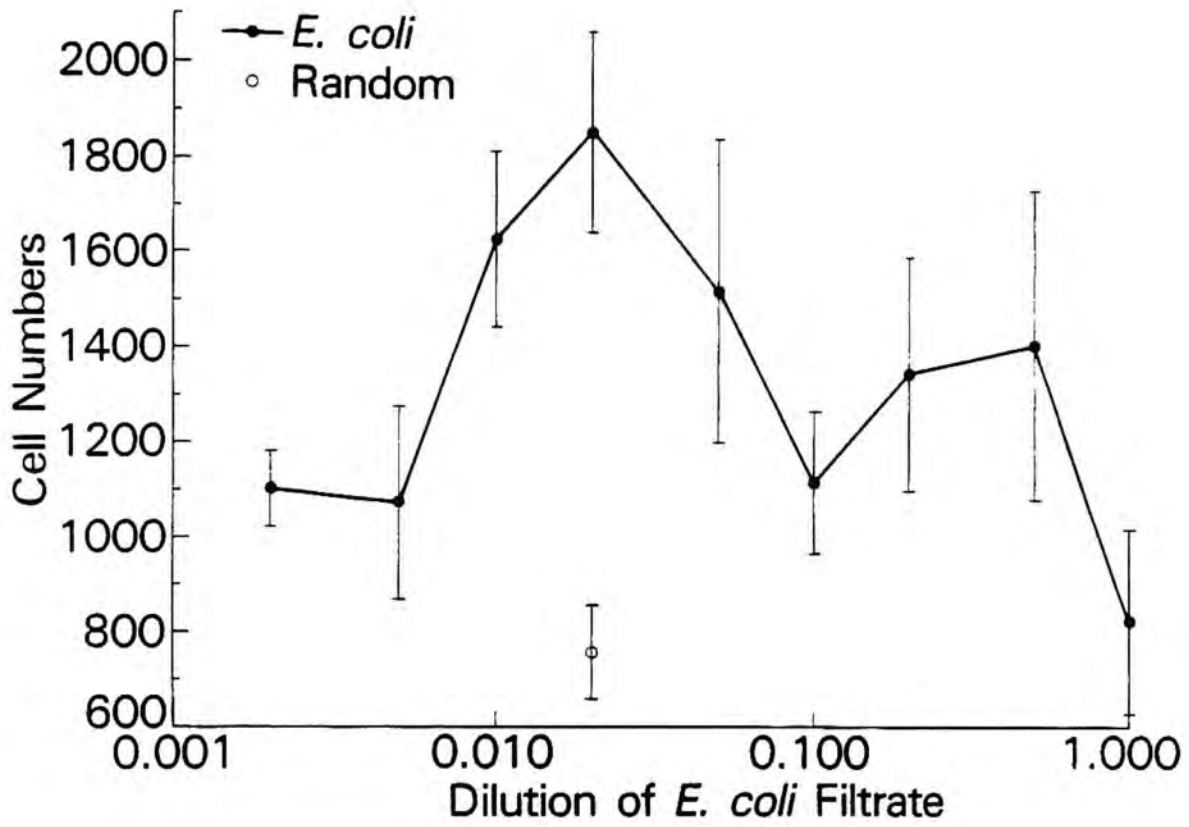




Figure 20. Migration of dog PMNs to E. coli filtrates. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . The sample size ranged from 4 to 11 animals.

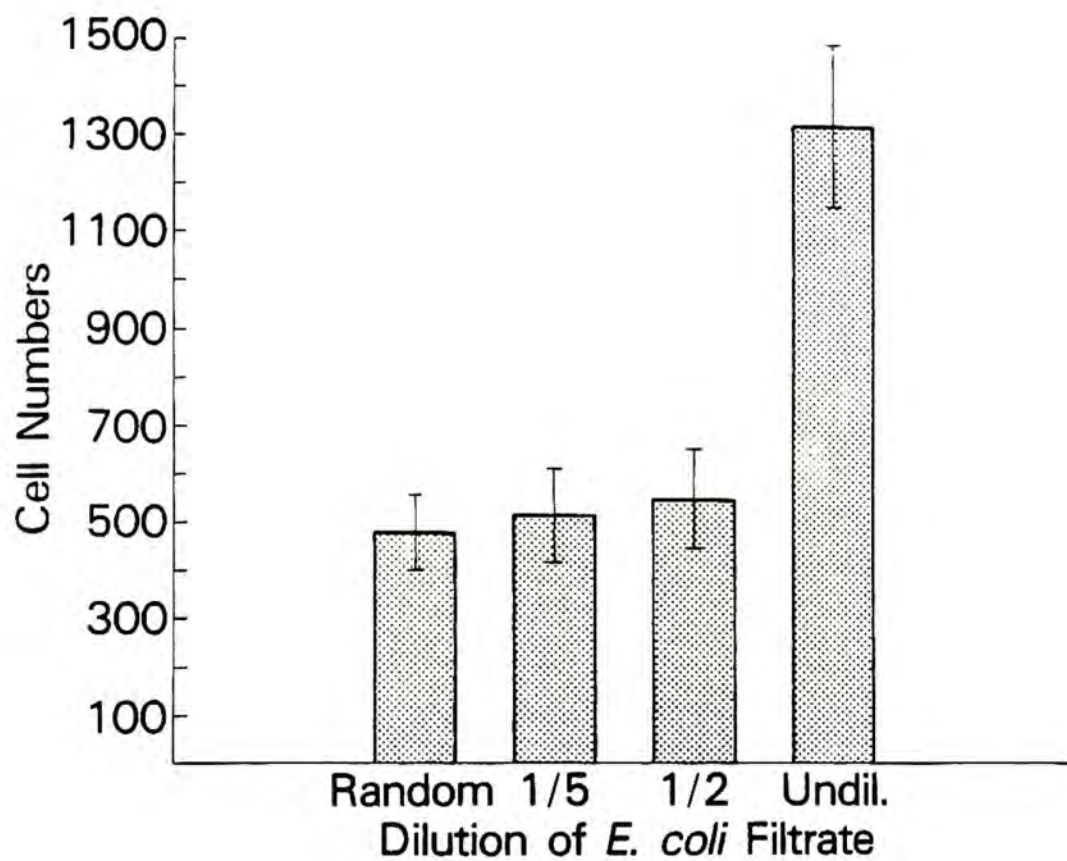


Figure 21. Inhibition of dog PMN migration to undiluted E. coli filtrate after preincubation for 120 minutes with 200 ng/ml of pertussis toxin. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . The sample size was 4 animals.



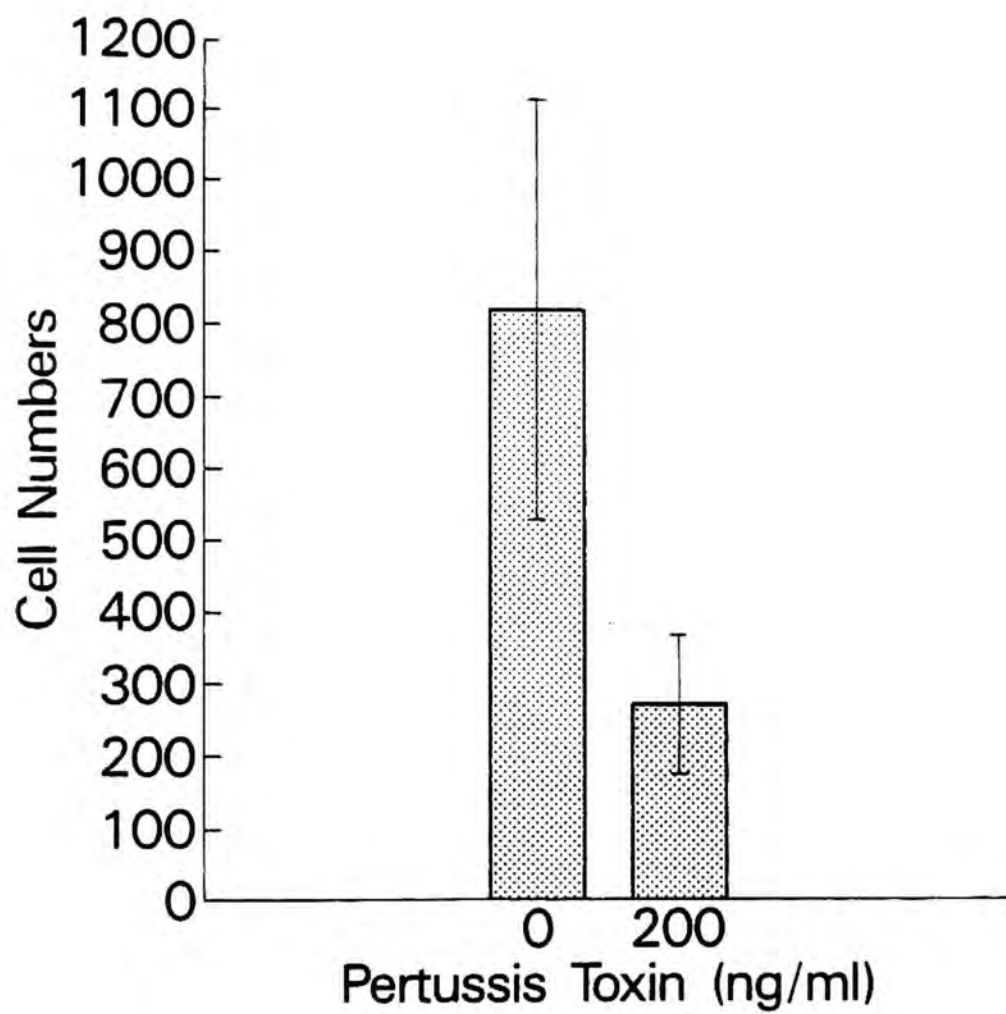


Figure 22. The effects of the formylpeptide receptor antagonist t-boc-MLP on human PMN migration to FMLP. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 6 subjects.

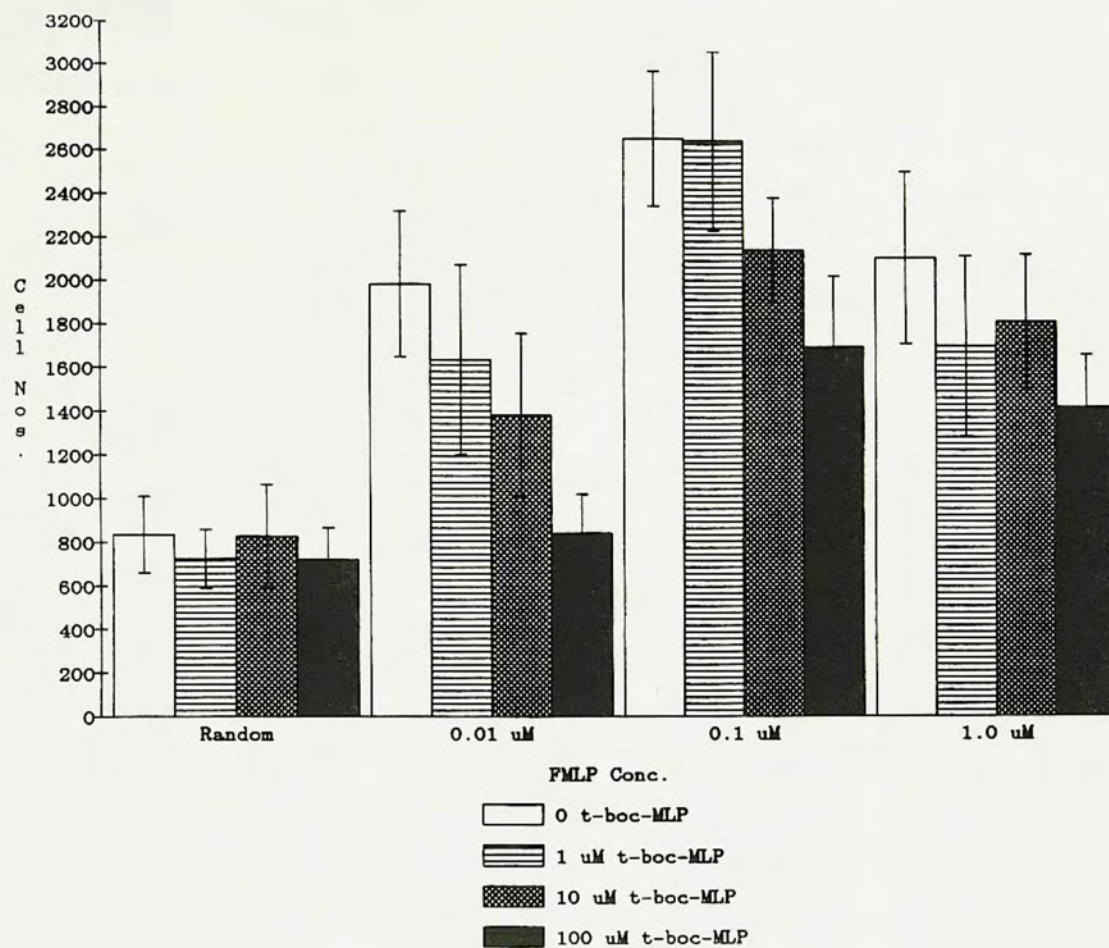


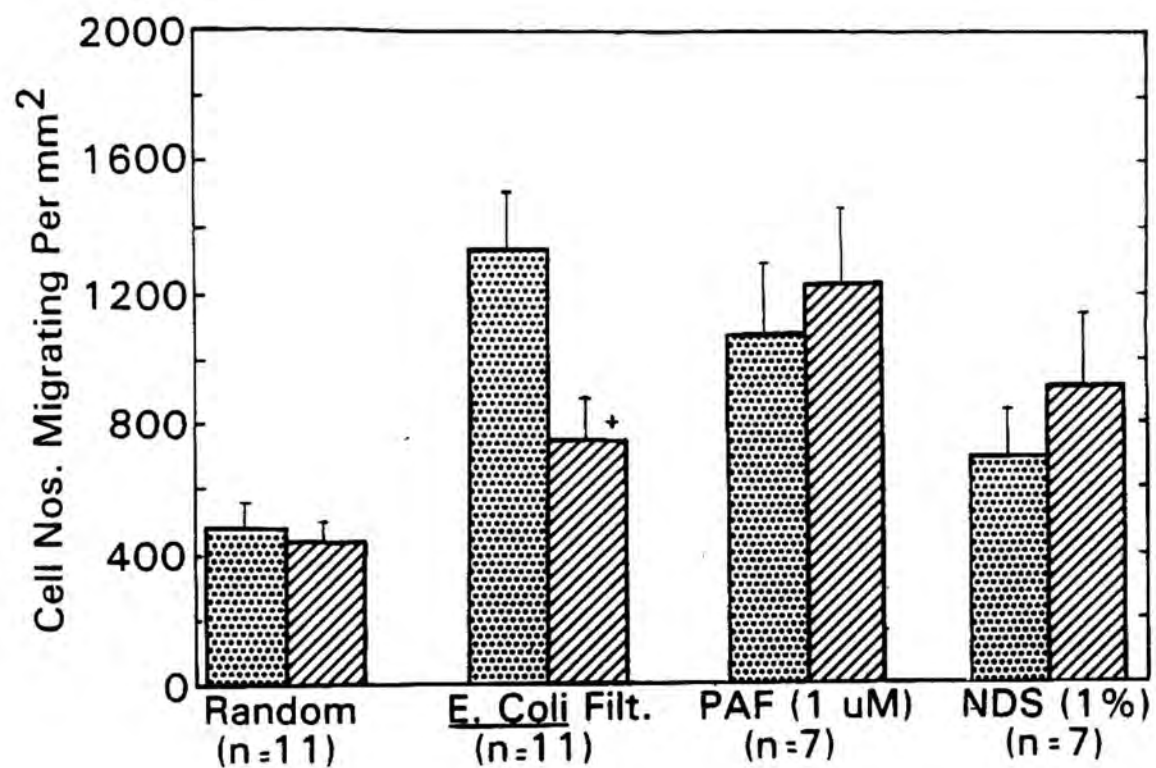




Figure 23. Effects of the presence ( ) or absence ( ) of 100 uM of the FMLP antagonist, t-boc-MLP on migration of dog PMNs to media, undiluted E. coli filtrate, 1 uM PAF or 1% NDS. All values are mean +/- SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size ranged from 7 to 11 animals. + represents significant differences between migration in the presence and absence of antagonist (P < 0.0001).



significantly changed (Figure 23). These data strongly suggest the dog PMN has formylpeptide receptors and that these receptors mediate migration to E. coli filtrate.

### 3. Responses of Dog PMNs to High Concentrations of FMLP

#### a. Migration of Dog PMNs to High Concentrations of FMLP

Results obtained from E. coli filtrates renewed our interest in dog PMN migration to FMLP at concentrations higher than tested in earlier studies. Dog PMNs were tested for motility to 0.001, 0.01, 0.1 and 1 mM FMLP (Figure 24). Migration to both 0.1 and 1 mM FMLP was significantly greater than random ( $P < 0.014$ ,  $P < 0.0001$  respectively) while there were no significant differences in migration at 1 or 10  $\mu$ M ( $P < 0.54$ ,  $P < 0.052$ ). At concentrations of FMLP stimulating maximum migration,  $9.65 \pm 0.9\%$  of the dog PMNs migrated and the stimulation ratio was  $1.9 \pm 0.26$ . The  $ED_{50}$  for migration to FMLP was  $1.05 \pm 0.10 \times 10^{-6}$  M. Control experiments demonstrated no differences between DMSO vehicle controls and random migration (Figure 24). Pretreatment of PMNs with 200 ng/ml of PT for 120 minutes inhibited migration to 1 mM FMLP by 56.8% (Figure 25).

#### b. Stimulation of the Oxidative Burst in Dog PMNs

To further characterize the unusual FMLP responses of dog PMNs, stimulation of oxidative metabolism by high concentrations of FMLP was investigated. Dog PMNs were exposed to 0.01, 0.1 and 1 mM FMLP in the presence and absence of cytochalasin B and assessed for NBT reduction. After exposure for 15 minutes to 1 mM FMLP,



Figure 24. The dose response curve of migration of dog PMNs to high concentrations of FMLP. Controls included corresponding dilutions of the DMSO vehicle and random migration. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 6 animals. \* represents migration significantly different from random, ( $P < 0.05$ ) and + represents migration significantly different from random, ( $P < 0.0001$ ).

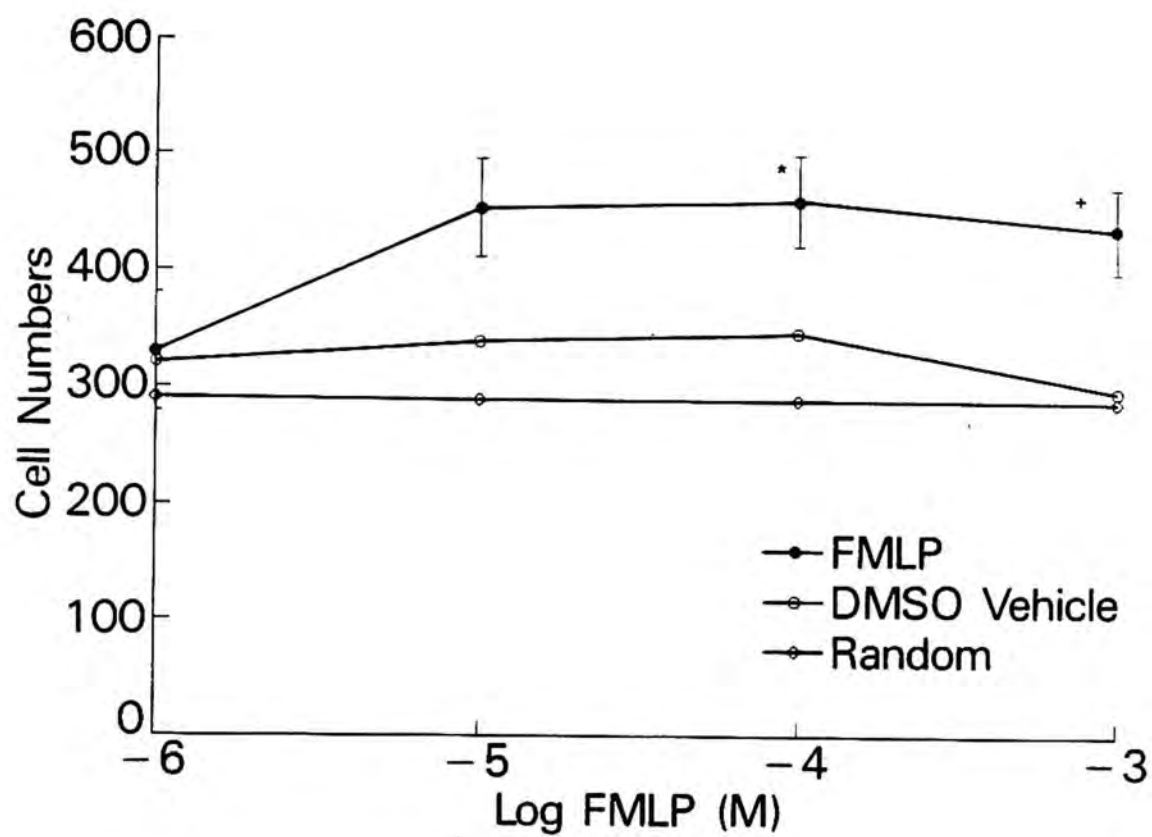
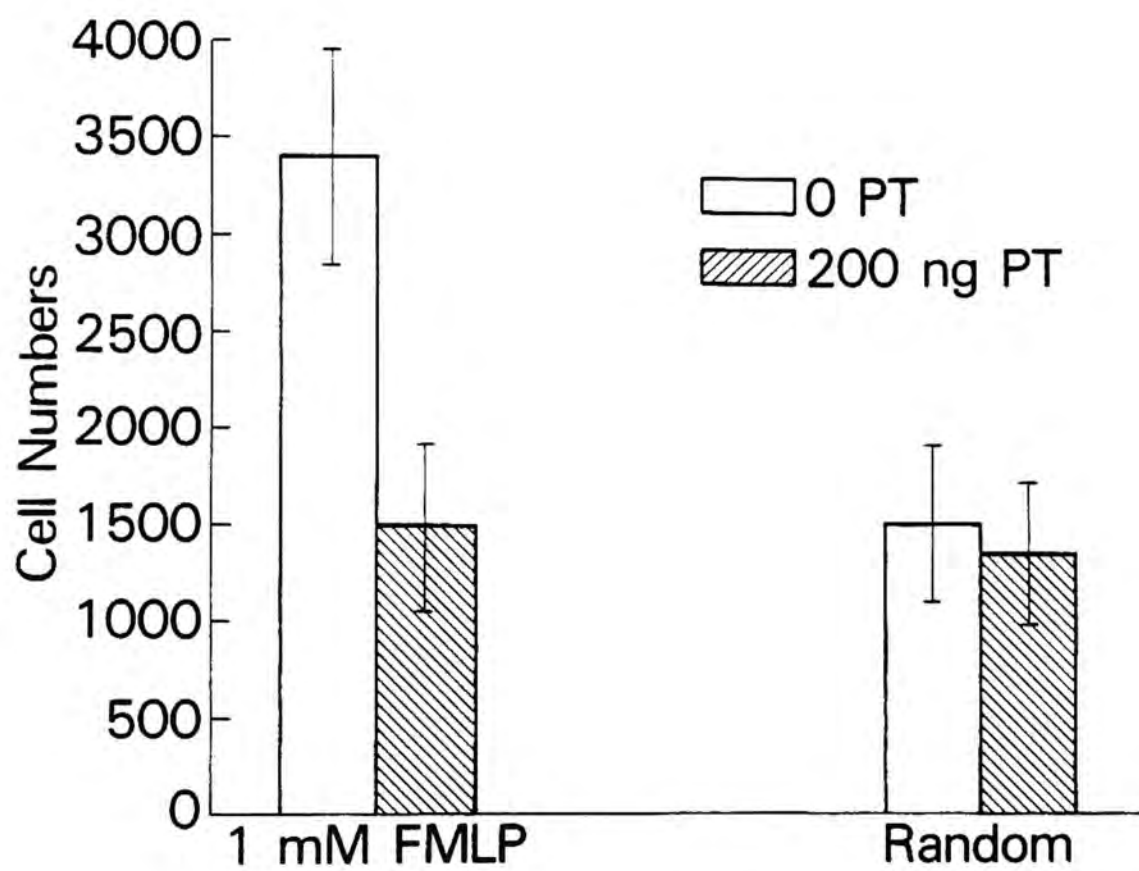


Figure 25. The effects of a 120 minute pretreatment of dog PMNs with 200 ng/ml pertussis toxin on migration to 1 mM FMLP or random migration. All values are the mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 6 animals.





23.91  $\pm$  8.92% for the media controls (Figure 26). In the presence of 65.3  $\pm$  13.45% of the PMNs were NBT positive as compared to cytoclasian B, greater than 90% of the PMNs were NBT positive at concentrations of FMLP of 0.1 and 1 mM.

#### 4. Response of Dog PMNs to Formylpeptide Analogs

The E. coli and FMLP data suggested receptors for formylpeptides on dog PMNs. Migration assays were next performed utilizing analogs with high affinity for the formylpeptide receptor. Based on a survey of the literature, the analogs FNLLPNLTL, FMLPP and FMLPB were chosen as potential stimulants. Figure 27 illustrates migration by dog PMNs in response to these stimulants compared to FMLP. FNLLPNLTL had an  $ED_{50}$  of 2.08  $\pm$  0.12  $\times 10^{-8}$  M for dog PMNs. FNLLPNLTL stimulated 12.9  $\pm$  2.9% of the PMNs to migrate with a stimulation ratio of 2.20  $\pm$  0.39. FMLPP elicited 18.8  $\pm$  2.1% of the PMNs to migrate while 19.0  $\pm$  2.5% migrated to FMLPB. The stimulation indices for these two analogs were 2.85  $\pm$  0.32 and 3.12  $\pm$  0.50 respectively, while the  $ED_{50}$  for FMLPP was 2.75  $\pm$  0.82  $\times 10^{-7}$  M and 8.61  $\pm$  3.7  $\times 10^{-7}$  M for FMLPB. These data are summarized in Table 5.

An analysis was performed to determine what percent of the migration to these compounds was chemokinetic. There were significant differences between chemotactic and chemokinetic migration to FMLP, FNLLPNLTL and FMLPB ( $P < 0.027$ , 0.047 and 0.045 respectively) while there was no difference between chemokinesis and chemotaxis to FMLPP. It should be noted that although the differences between these types of

Figure 26. NBT reduction by dog PMNs in response to high concentrations of FMLP in the presence and absence of 5 ug/ml cytochalasin B. All values are the mean  $\pm$  SEM and represent percentages of NBT positive PMNs. The sample size was 6 animals.



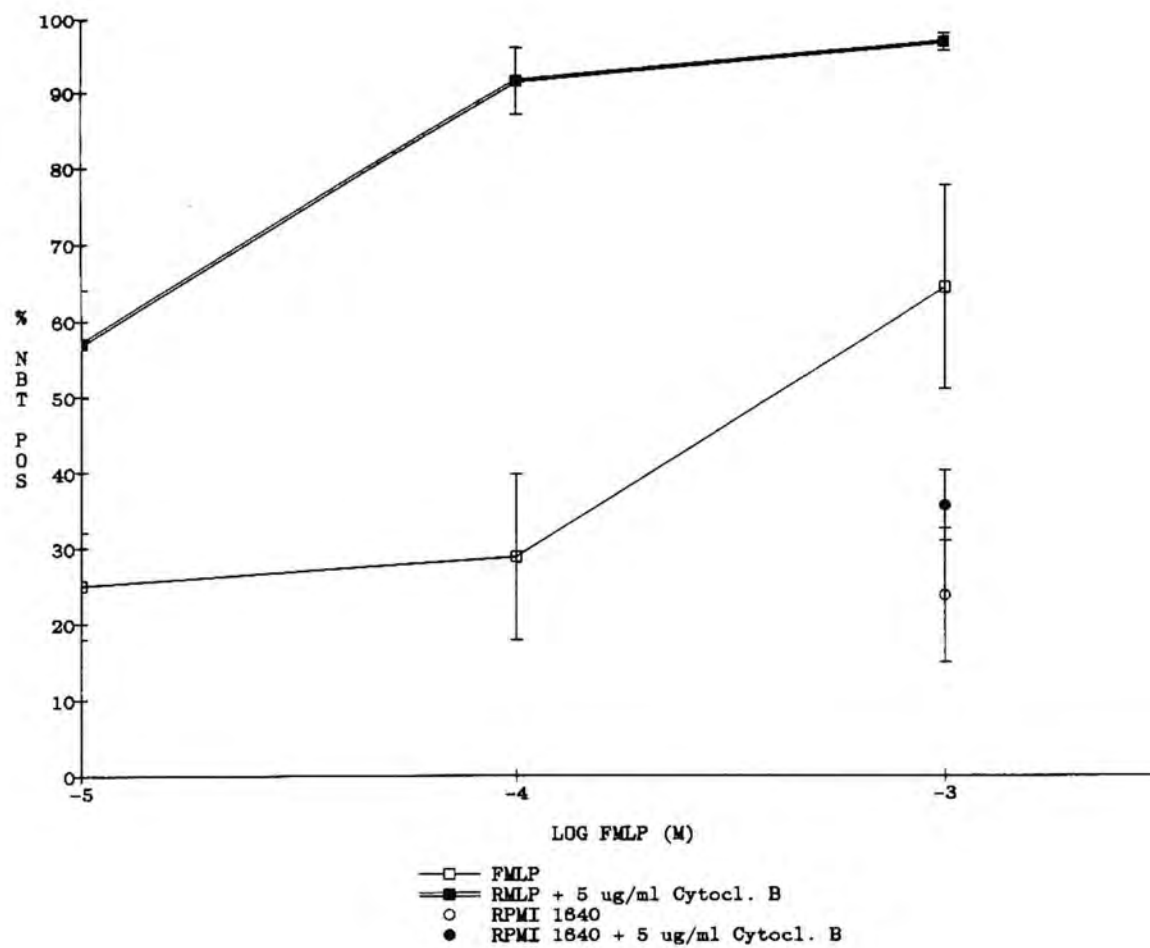


Figure 27. The dose response curve of dog PMN migration to FMLP and the high affinity formylated peptide analogs FMLPP, FMLPB and FNLLPNLTL. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 6 to 8 animals.

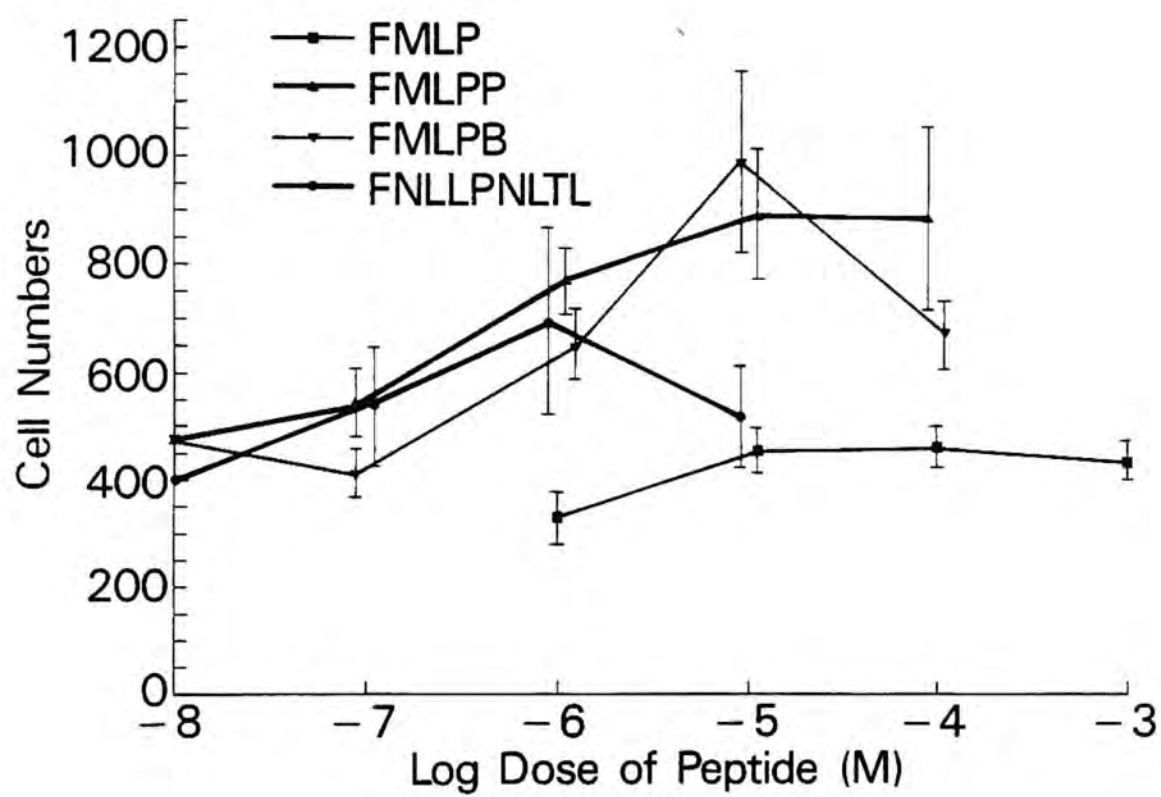


TABLE 5

## SUMMARY OF MIGRATION RESPONSES TO FORMYLATED PEPTIDE ANALOGS BY DOG PMNs

Analog	ED <sub>50</sub>	% PMNs Migr.	Ratio
FNLLPNLTL	2.08 +/- 0.12 X 10 <sup>-8</sup> M	12.90 +/- 2.88%	2.20 +/- 0.39
FMLPP	2.75 +/- 0.82 X 10 <sup>-7</sup> M	18.80 +/- 2.13%	2.85 +/- 0.32
FMLPB	8.61 +/- 3.70 X 10 <sup>-7</sup> M	19.00 +/- 2.45%	3.12 +/- 0.50
FMLP	1.05 +/- 0.10 X 10 <sup>-6</sup> M	9.65 +/- 0.90%	1.90 +/- 0.26

All values represent mean +/- SEM



motility was statistically significant, chemokinesis was often a high percentage of the motility observed. This relationship is depicted in Figure 28. Interestingly chemokinesis was 47% of motility using FMLPB while for FMLP, FNLLNLTL and FMLPP, chemokinesis was over 85% of total migration. Figure 29 illustrates the effects of PT on migration to FMLPP and FMLPB. FMLPP migration was inhibited 56.2% when exposed to 200 ng/ml PT for 120 minutes and 82.3% after exposure to 500 ng/ml. FMLPB was inhibited 52.4% and 88.6% after 120 minutes pretreatment with 200 and 500 ng/ml PT, respectively (Figure 29).

### C. RHESUS MONKEY STUDIES

#### 1. Overview

The capacity for rhesus monkey PMNs to respond to FMLP was assessed after treatment with rhGM-CSF in vitro and in vivo. Studies were performed evaluating the effects of rhGM-CSF on FMLP induced migration and reactive oxygen intermediate production as well as expression of formylpeptide receptors by PMNs obtained from the peripheral blood of rhesus monkeys.

#### 2. In Vitro Modulation of Functional Responses

##### a. Effects of In Vitro Pretreatment with rhGM-CSF on

##### Rhesus Monkey PMN Migration to FMLP

The time and dose dependent increase in migration of normal monkey PMNs to FMLP resulting from in vitro rhGM-CSF pretreat-

Figure 28. Representation of the chemokinetic component of dog PMN migration to FMLP or the high affinity formylated peptide analogs FMLPP, FMLPB and FNLLPNLTL. All values are mean  $\pm$  SEM and represent numbers of PMNs migrating per  $\text{mm}^2$ . Sample size was 5 to 10 animals.

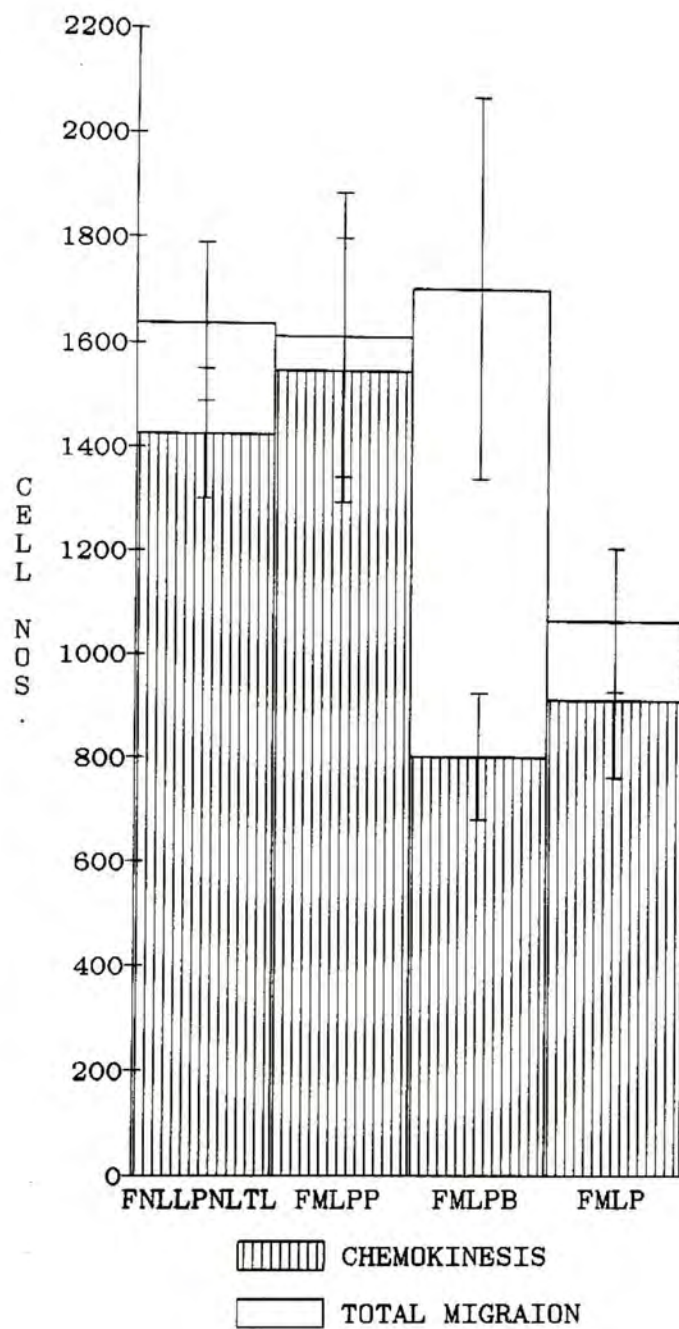
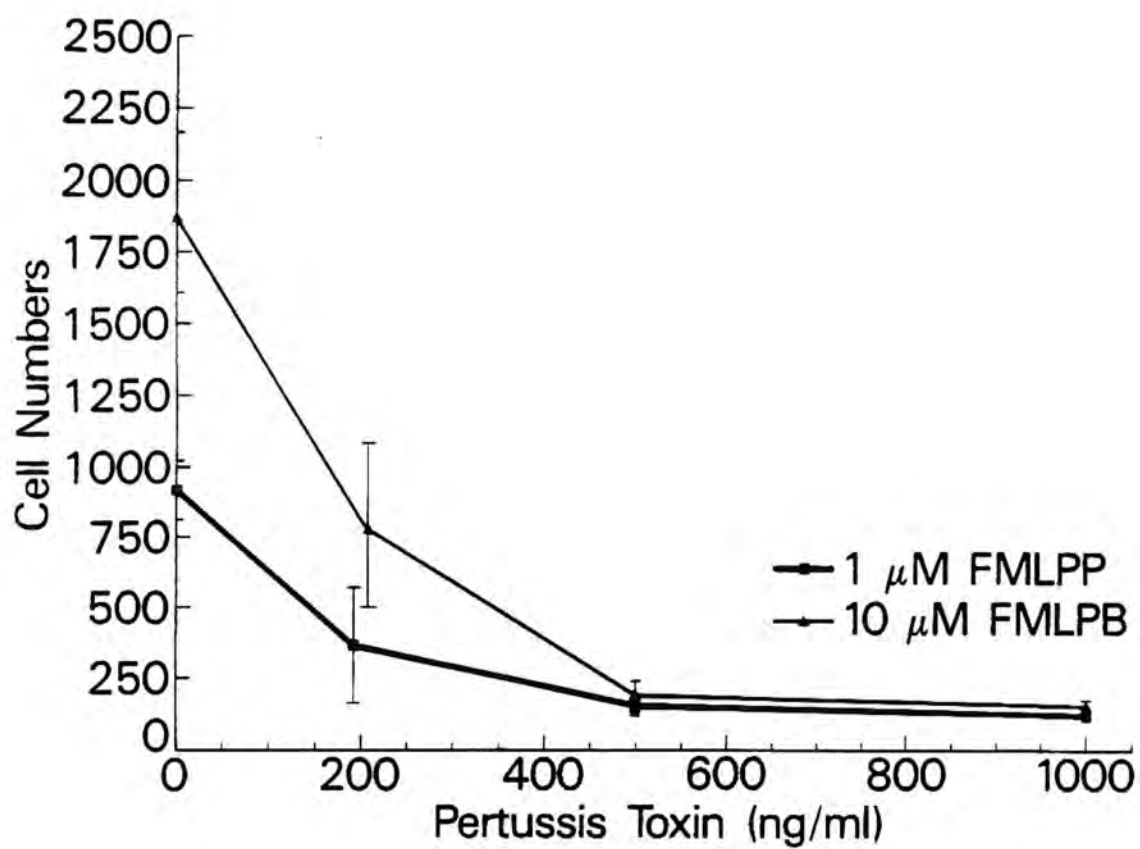


Figure 29. The effects of a 120 minute pretreatment of dog PMNs with varied concentration of pertussis toxin on migration to FMLPP and FMLPB. All values are the mean  $\pm$  SEM and represent numbers of PMNs migrating per mm<sup>2</sup>. Sample size was 3 animals.





ment is depicted in Figure 30. The migration of PMNs pretreated 5 minutes with 100 pM (10 U/ml) rhGM-CSF was significantly ( $P < 0.05$ ) greater than controls. Treatment with 1000 pM (100 U/ml) of rhGM-CSF increased migration to FMLP but results were more variable at this dose than those at 100 pM. Migration to FMLP returned to normal after 15 to 60 minutes of pretreatment. Figure 31 illustrates the dose related increase in migration to both FMLP and PAF after 5 minutes pretreatment with rhGM-CSF at concentrations of 1, 10, and 100 U/ml (10, 100, and 1000 pM, respectively). A 5 minute pretreatment of PMNs with rhGM-CSF enhanced migration to PAF although there was variability in the magnitude of the donor response (Figure 31). Random migration was not altered significantly in these in vitro studies.

#### b. Effects of rhGM-CSF on FMLP Stimulated Oxidative Burst Generation

Rhesus monkey PMNs pretreated with rhGM-CSF for 5, 15, 60 and 120 minutes were assessed for ability to respond to 0.1  $\mu$ M FMLP using the NBT assay. The results from these experiments are depicted in Figure 32. Pretreatment for 120 minutes resulted in significant increases ( $P < 0.003$ ) in the numbers of NBT positive PMNs. Consequently, all subsequent studies were conducted pretreating rhesus monkey PMNs with rhGM-CSF for 120 minutes prior to FMLP exposure. Figure 33 illustrates that the 120 minute pretreatment did not cause an increase in activation in the absence of a stimulant, as demonstrated by the media controls ( $15.5 \pm 5.2$  compared to  $16.1 \pm 2.5$ ), or in the basal response to FMLP ( $32.3 \pm 4.9$  versus

Figure 30. The effect of in vitro treatment with rhGM-CSF on migration of rhesus monkey PMNs to 0.1  $\mu$ M FMLP. PMNs were pretreated for 0, 5, 15 and 60 minutes with 0, 1, 10, and 100 U/ml of rhGM-CSF. Data are expressed as the percent difference between responses of control cells incubated with no rhGM-CSF and those in the presence of rhGM-CSF at corresponding timepoints. Each value is the mean  $\pm$  SEM of 3 animals. \* represents a significant difference in mean percent change from 0, ( $P < 0.05$ ).

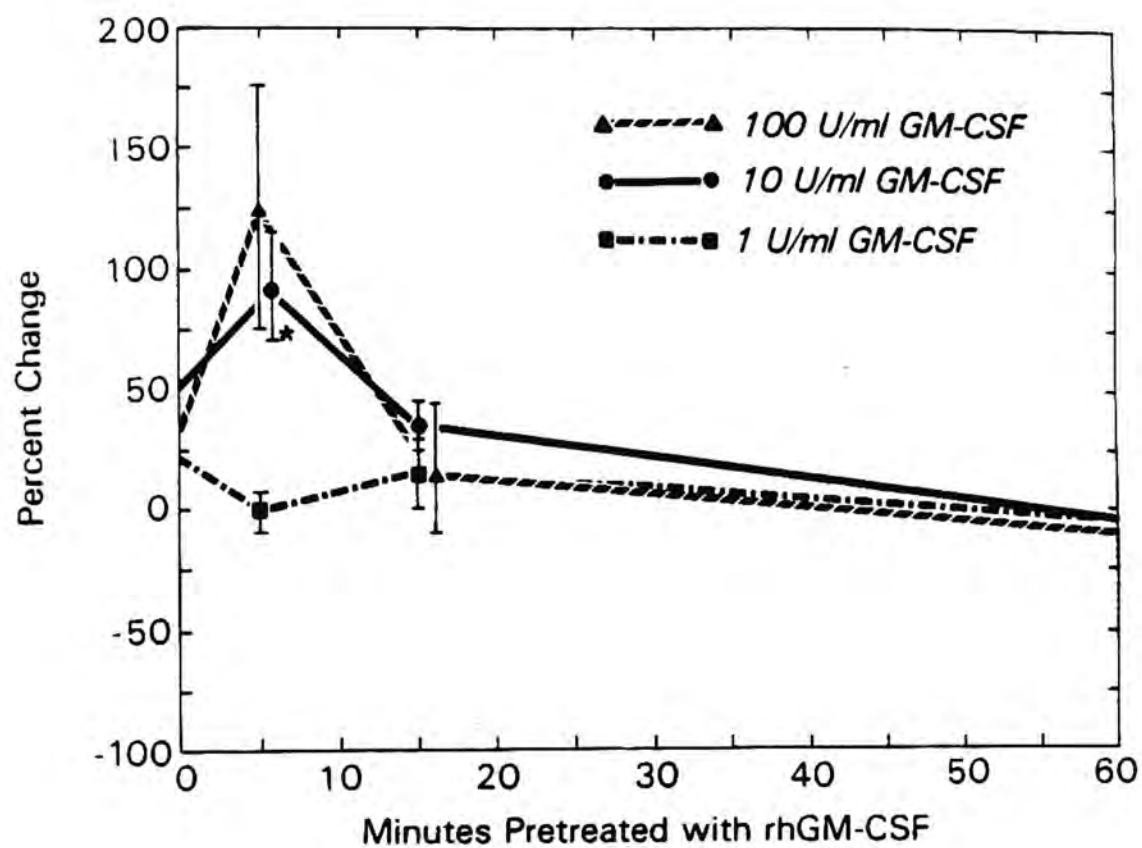




Figure 31. The dose related effects of a 5 minute in vitro treatment of rhesus monkey PMNs with rhGM-CSF on random migration as well as migration to 0.1 uM FMLP and 1 uM PAF. Data are expressed as the percent difference between responses of cells incubated with no rhGM-CSF and those in the presence of rhGM-CSF at corresponding timepoints. Each value is the mean of three animals. \* represents a significant difference in mean percent change from 0, ( $P < 0.05$ ).

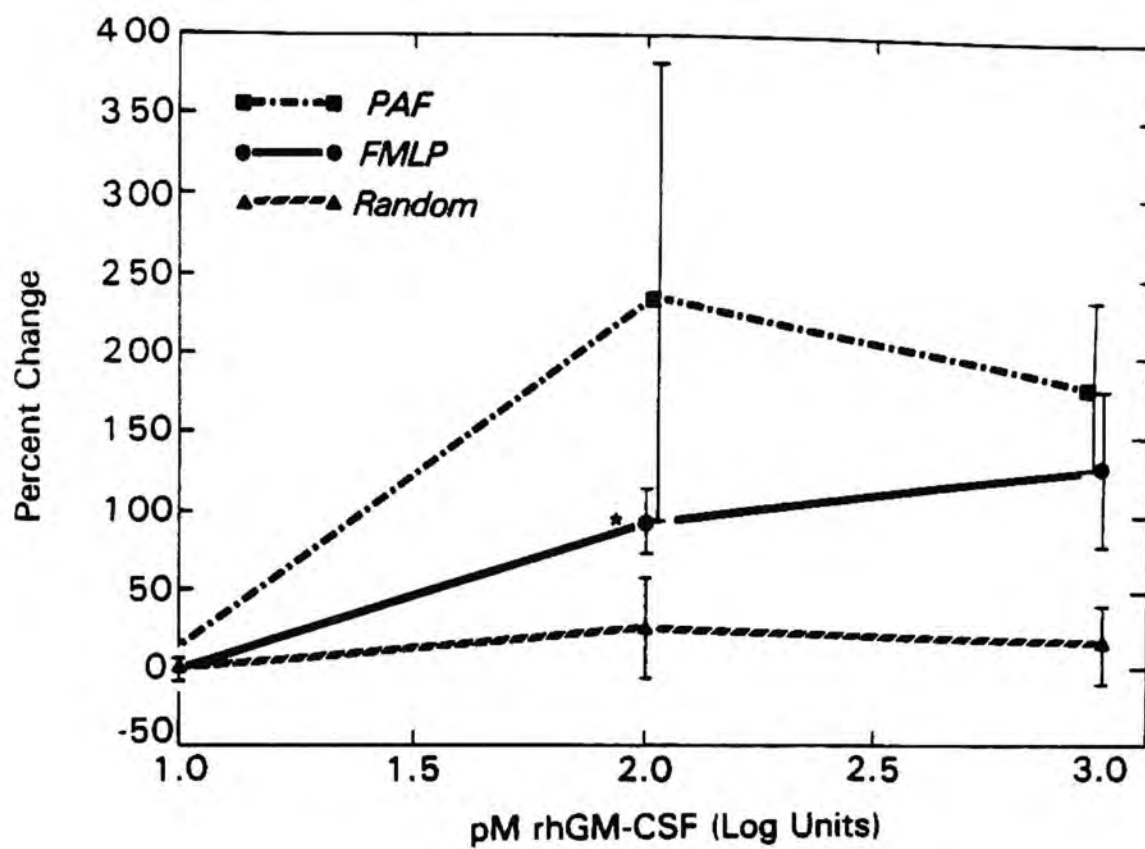


Figure 32. The effects of in vitro pretreatment of rhesus monkey PMNs with 10 U/ml of rhGM-CSF for 5, 15, 60 and 120 minutes on NBT reduction in response to 0.1  $\mu$ M FMLP. All values are the mean  $\pm$  SEM and represent percentages of NBT positive PMNs. \*\*\* represents a significant difference in percent NBT positive PMNs after rhGM-CSF pretreatment for 120 minutes when compared to NBT positive PMNs incubated 120 minutes in the absence of rhGM-CSF ( $P < 0.005$ ).

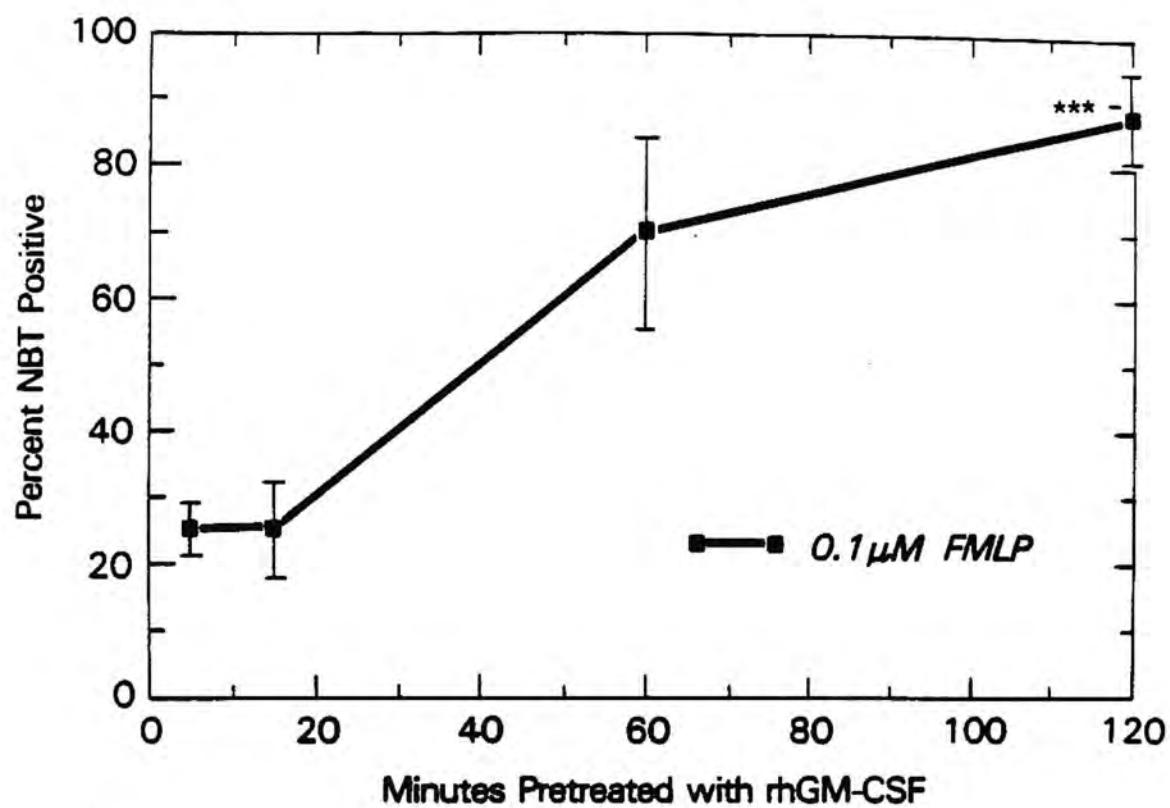
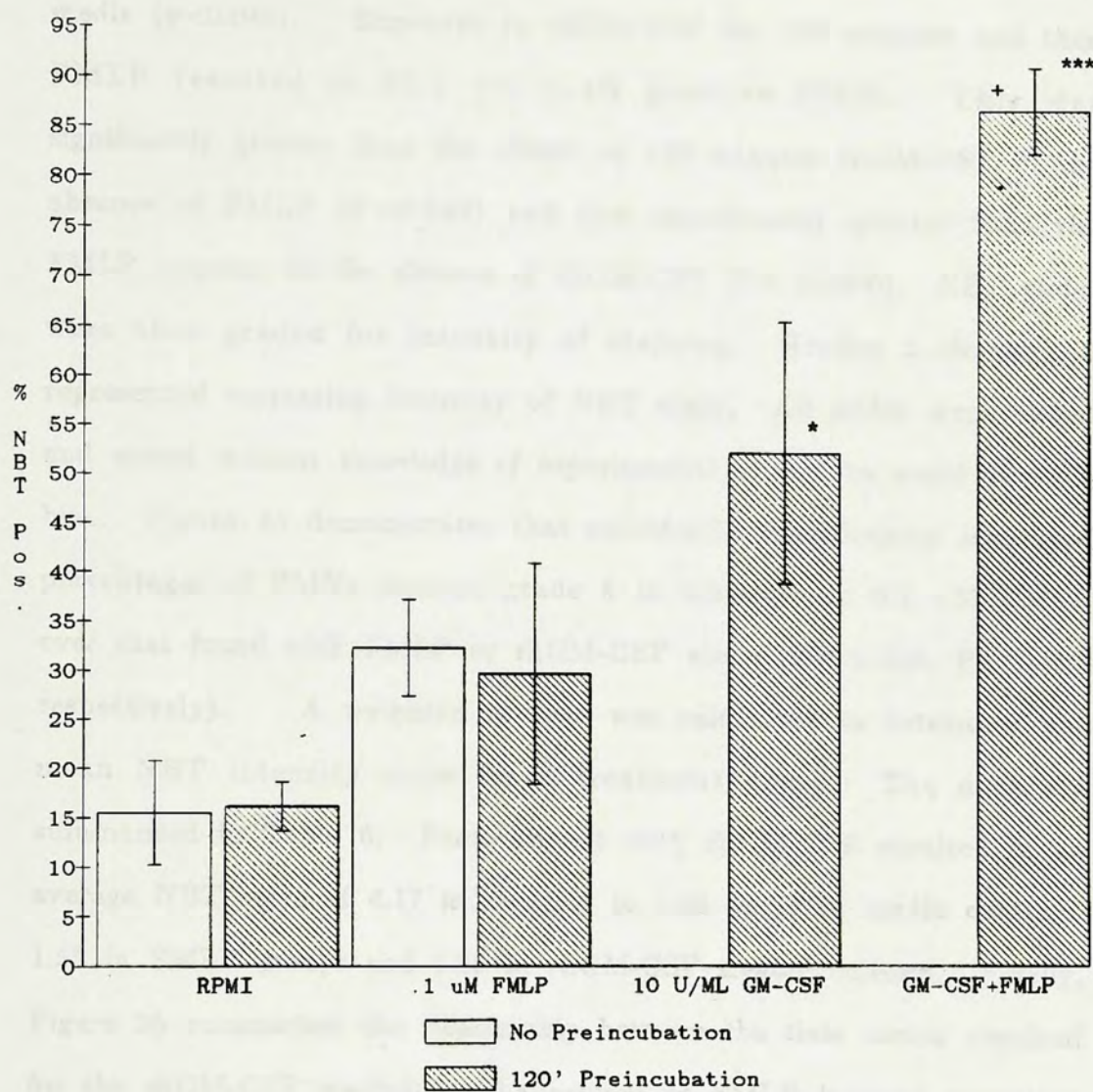




Figure 33. The effects of a 120 minute preincubation in the presence or absence of 10 U/ml rhGM-CSF on percentages of NBT positive rhesus monkey PMNs in response to either media controls (RPMI 1640) or 0.1 uM FMLP. PMNs were obtained from the peripheral blood of normal rhesus monkeys. All values are the mean  $\pm$  SEM. \* represents a significant difference between percentages of NBT positive PMNs after a 120 minute pretreatment with rhGM-CSF from corresponding media controls, ( $P < 0.05$ ); \*\*\* represents a significant difference between percentages of NBT positive PMNs after a 120 minute pretreatment with 10 U/ml rhGM-CSF in the presence of 0.1 uM FMLP and percentages of NBT positive PMNs pretreated 120 minutes without rhGM-CSF in the presence of 0.1 uM FMLP ( $P < 0.0006$ ); + represents a significant difference between percentages of NBT positive PMNs after 120 minutes pretreatment with rhGM-CSF in the presence of 0.1 uM FMLP and percentages of NBT positive PMNs after 120 minutes pretreatment with rhGM-CSF in the absence of 0.1 uM FMLP ( $P < 0.05$ ).





29.8  $\pm$  11.3). Interestingly, pretreatment with rhGM-CSF for 120 minutes led to 52.3  $\pm$  13.3% of the PMNs becoming NBT positive, an increase significantly greater than 120 minutes incubation with media ( $p < 0.045$ ). Exposure to rhGM-CSF for 120 minutes and then FMLP resulted in 87.1  $\pm$  4.4% positive PMNs. This was significantly greater than the effects of 120 minutes rhGM-CSF in the absence of FMLP ( $P < 0.048$ ) and also significantly greater than the FMLP response in the absence of rhGM-CSF ( $P < 0.0006$ ). NBT slides were then graded for intensity of staining. Grades 1 through 5 represented increasing intensity of NBT stain. All slides were coded and scored without knowledge of experimental groups to avoid possible bias. Figure 34 demonstrates that rhGM-CSF significantly increased percentages of PMNs stained grade 5 in response to 0.1  $\mu$ M FMLP over that found with FMLP or rhGM-CSF alone ( $P < 0.036$ ,  $P < 0.003$  respectively). A weighted average was calculated to determine the mean NBT intensity score for a treatment group. The data are summarized in Table 6. Pretreatment with rhGM-CSF resulted in an average NBT score of 4.17 in contrast to 1.38 found in media controls, 1.55 in FMLP groups and 1.59 in rhGM-CSF treated cultures. Finally, Figure 35 summarizes the relationship between the time course required for the rhGM-CSF mediated enhancement of FMLP induced migration and oxidative metabolism responses. Thus, in vitro pretreatment of rhesus monkey PMNs with rhGM-CSF rapidly enhanced migration to FMLP while longer exposures increased numbers of PMNs generating a respiratory burst.

Figure 34. The effects of a 120 minute pretreatment with media or 10 U/ml of rhGM-CSF on staining intensity of NBT positive rhesus monkey PMNs. PMNs were pretreated with media or 10 U/ml of rhGM-CSF for 120 minutes and then exposed for 15 minutes to either media or 0.1  $\mu$ M FMLP. Slides were graded for intensity of staining. Grade 1 was the lightest stain and grade 5 was the darkest stain. All values are the mean  $\pm$  SEM and represent percent NBT positive PMNs. \* represents significant differences from percentages of PMNs stained grade 5 in response to 0.1  $\mu$ M FMLP, ( $P < 0.05$ ) and \*\* represents significant differences from percentages of PMNs stained grade 5 in response to 120 minutes of rhGM-CSF treatment, ( $P < 0.01$ ).



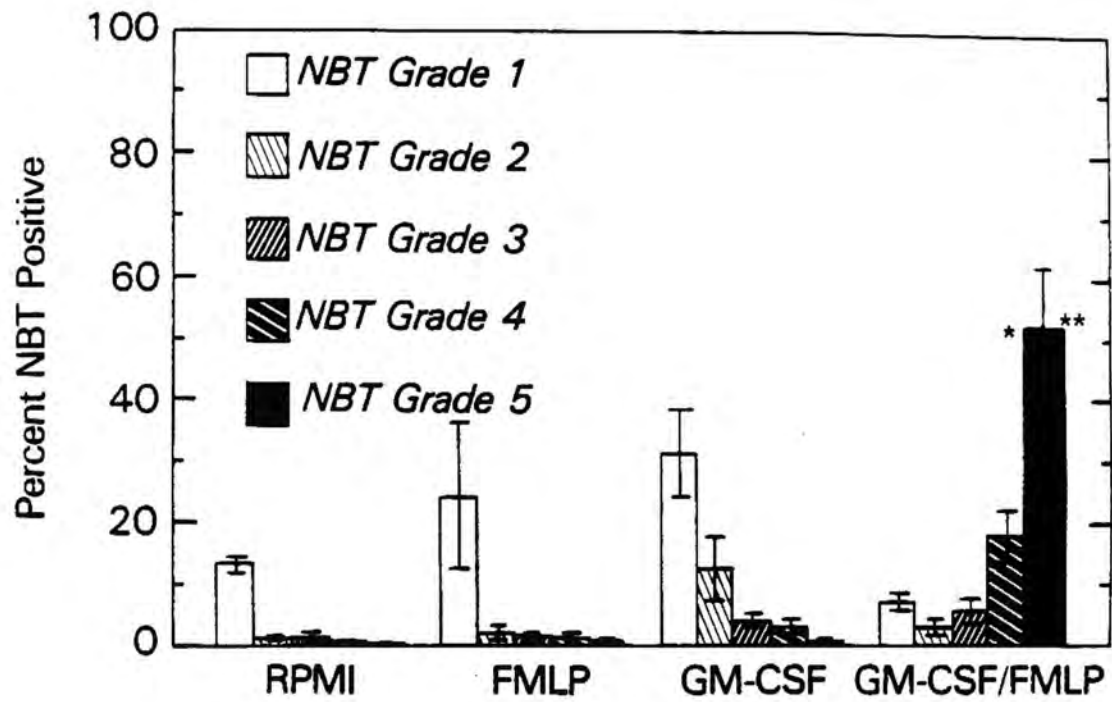


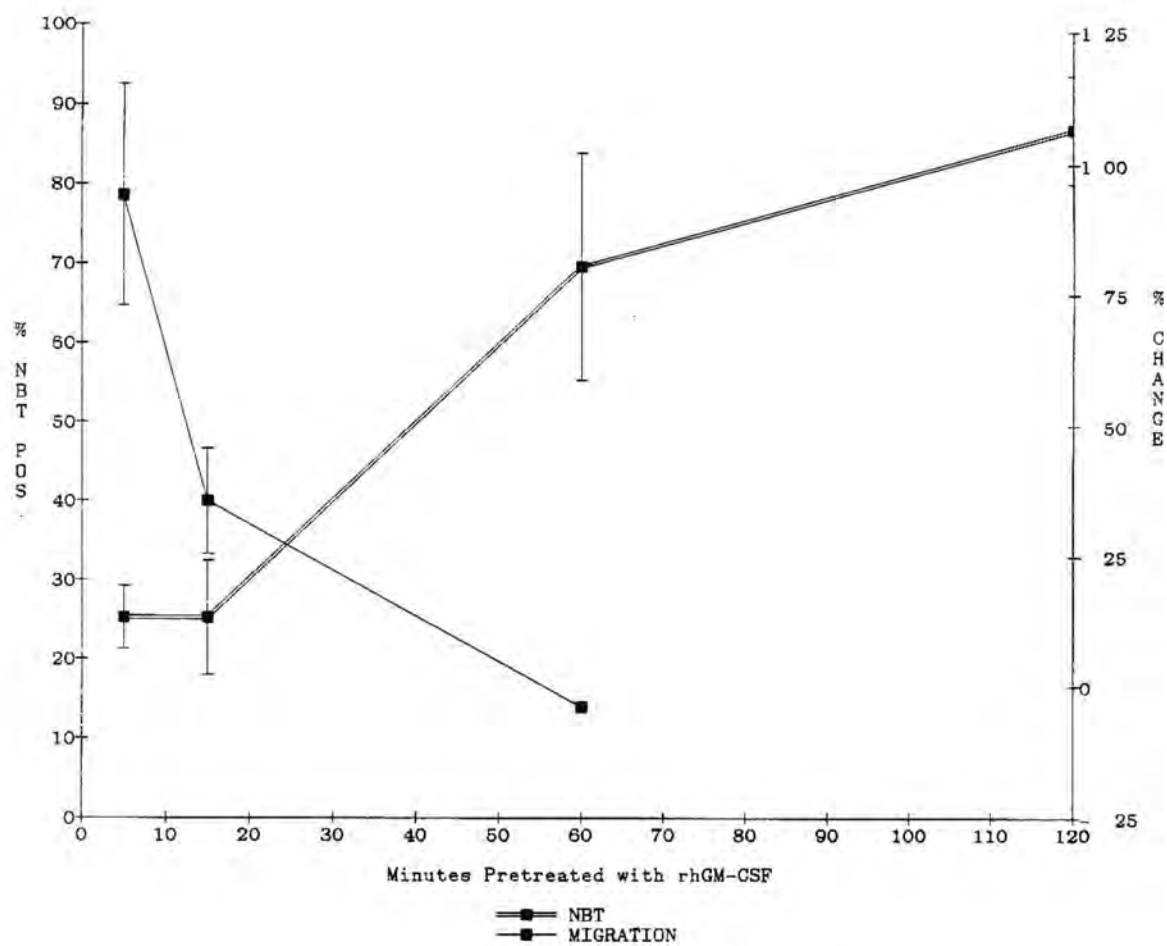
TABLE 6

SUMMARY OF MEAN STAINING INTENSITY SCORES FOR NBT  
POSITIVE RHESUS MONKEY PMNs

Treatment	% NBT Positive	Mean NBT Intensity Score
RPMI 1640	16.1 +/- 2.4	1.38
FMLP (0.1 uM)	30.0 +/- 11.3	1.55
rhGM-CSF (10 U/ml)	52.3 +/- 13.3	1.59
rhGM-CSF (10 U/ml)+ FMLP (0.1 uM)	87.1 +/- 4.4	4.17

All values represent means +/- SEM

Figure 35. The kinetics of rhGM-CSF recruitment of PMN response to 0.1  $\mu$ M FMLP. PMNs obtained from normal rhesus monkey peripheral blood were pretreated for 5, 15, 60 and 120 minutes with 10 U/ml rhGM-CSF and then assessed for migration or oxidative burst in response to 0.1  $\mu$ M FMLP. Migration data are expressed as the percent difference between responses of control cells incubated with no rhGM-CSF and those in the presence of rhGM-CSF at corresponding timepoints. Each value is the mean  $\pm$  SEM. NBT data are expressed as percentages of NBT positive PMNs and represent the mean  $\pm$  SEM.



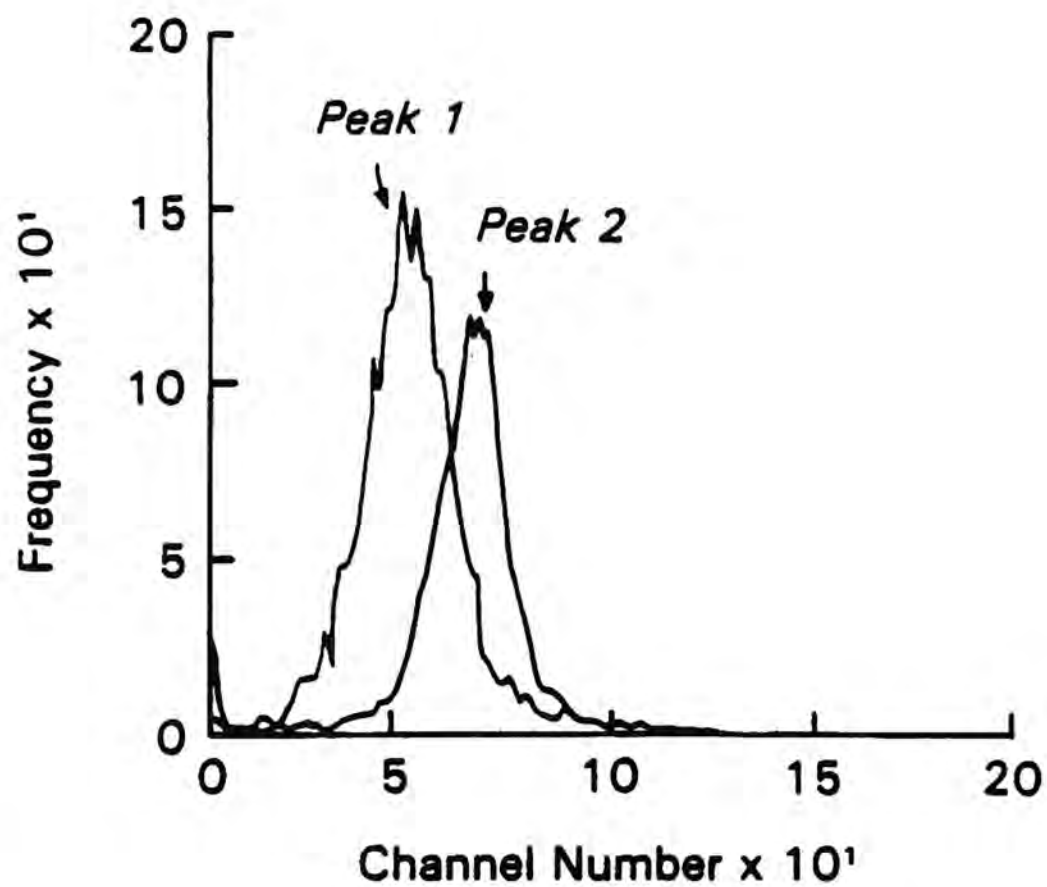


Studies were also performed assessing the effects of rhGM-CSF pretreatment on FMLP elicited  $H_2O_2$  production. PMNs isolated from normal monkeys were pretreated with rhGM-CSF for 120 minutes in a  $37^{\circ}C$  incubator, loaded with DCF-DA and stimulated with 10  $\mu M$  FMLP for 15 minutes. DCF-DA is converted to DCF-H in the cytoplasm. DCF-H is nonfluorescent and is oxidized to the fluorescent molecule DCF by  $H_2O_2$ . Comparisons were made in mean channel fluorescence (ie, intensity of the fluorescence) between baseline ( $T=0$ ) and stimulated ( $T=15$ ) timepoints. As discussed, 10  $\mu M$  FMLP increased fluorescence by 104% and 117% in the two monkeys tested. Figure 7 depicts responses of PMNs to 10  $\mu M$  FMLP from a representative monkey. Similar responses were noted after exposure to 0.1  $\mu M$  FMLP. To determine the effects of rhGM-CSF treatment on FMLP stimulated  $H_2O_2$  production, PMNs were pretreated with rhGM-CSF for 120 minutes and then stimulated with FMLP. Pretreatment for 120 minutes with rhGM-CSF (prior to FMLP stimulation) resulted in increased fluorescence in each of the four monkeys evaluated. A representative histogram illustrating these effects is shown in Figure 36. The increased fluorescence resulting from rhGM-CSF pretreatment alone made evaluation of the resulting changes in FMLP response difficult. Four of six monkeys tested had a higher mean channel fluorescence after rhGM-CSF/FMLP treatment than those exposed only to FMLP (data not shown).

### c. Effects of rhGM-CSF on Formylpeptide Receptor Expression

Binding studies were conducted to evaluate if enhanced migration and oxidative burst responses correlated with increases in FMLP

Figure 36. The effect of a 120 minute pretreatment with rhGM-CSF on mean fluorescent intensity (represented by mean channel number) of DCF loaded rhesus monkey PMNs. PMNs were loaded with 2'7' dichlorofluorescein diacetate (DCF-DA), which is taken up through the membrane. DCF-DA is deacetylated to 2'7' dichlorofluorescein (DCF-H). DCF-H is rapidly oxidized to the fluorescent form, 2'7' dichlorofluorescein (DCF) in the presence of  $H_2O_2$ . Increases in fluorescent intensity are proportional to the amount of  $H_2O_2$  produced. Peak 1 represents PMNs treated 120 minutes with RPMI 1640 while peak 2 represents PMNs treated 120 minutes with rhGM-CSF.



binding. PMNs isolated from two normal monkeys were pretreated with rhGM-CSF for 5, 15 and 90 minutes and then assessed for binding of  $^3\text{H}$ -FMLP. No increases in specific binding of FMLP were observed (data not shown). However, due to the limited sensitivity of the binding assay changes in receptor expression can not definitively be ruled out.

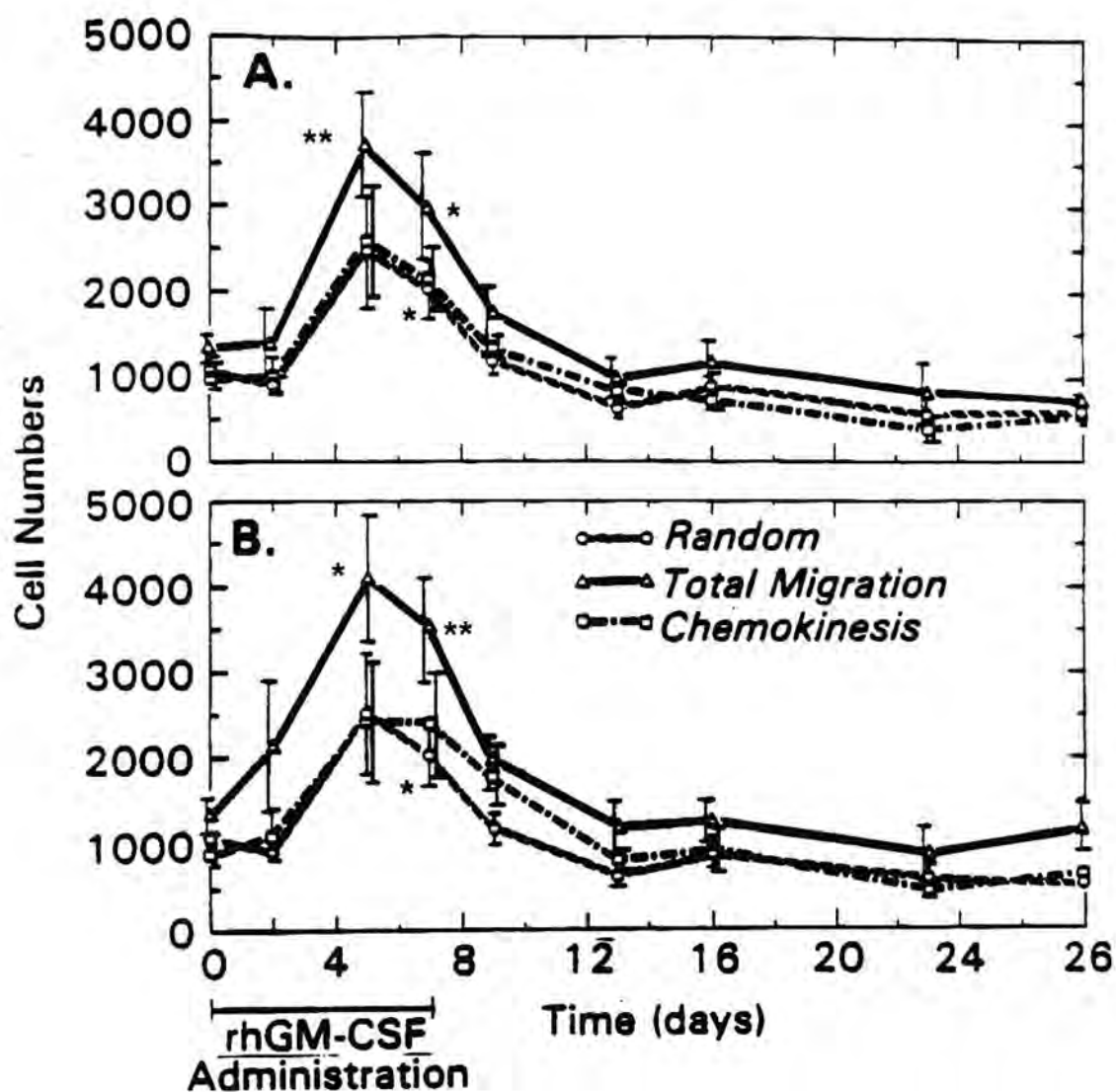
### 3. In Vivo Modulation of Rhesus Monkey PMN Functional Responses

#### a. Effects of In Vivo rhGM-CSF Administration on Migration of Rhesus Monkey PMNs to FMLP

PMNs collected from monkeys infused with rhGM-CSF for a seven day period were evaluated for their ability to migrate to 0.1  $\mu\text{M}$  FMLP and 1  $\mu\text{M}$  PAF. PMN migration to FMLP and PAF (Figure 37 A and B) was significantly above baseline values on day 5 ( $P < 0.01$  for FMLP,  $P < 0.02$  for PAF) and day 7 ( $P < 0.03$  for FMLP,  $P < 0.01$  for PAF). PMN migration to PAF was elevated on days 2 and 9, although not significantly, and returned to normal on day 13 (Figure 37 B). PMN migration to FMLP returned to normal by day 13 (Figure 37 A). Both Figure 37 A and B show random migration was elevated on day 5 and significantly greater than baseline values on day 7 of rhGM-CSF treatment ( $P < 0.02$ ). Chemokinetic increases for both stimulants followed the trends in random migration (Figures 37 A and 37 B). PMNs from control monkeys implanted with saline filled pumps had no changes in migration to FMLP and PAF during the evaluation period (data not shown).



Figure 37. Effects of a continuous infusion of rhGM-CSF on migration of rhesus monkey PMNs. PMNs obtained from the peripheral blood of monkeys receiving rhGM-CSF over a seven day period were assessed for random migration as well as migration to 0.1  $\mu$ M FMLP (Panel A) and 1  $\mu$ M PAF (Panel B). Total migration and chemokinesis are illustrated for each stimulant. Data are expressed as numbers of PMNs migrating per  $\text{mm}^2$  and represent the mean  $\pm$  SEM of 6 animals. \* represents migration significantly different from baseline, ( $P < 0.05$ ) and \*\* represents migration significantly different from baseline, ( $P < 0.01$ ).



b. Effects of In Vivo rhGM-CSF Administration on PMN Responses  
to rhGM-CSF In Vitro

PMNs isolated from the peripheral blood of rhesus monkeys administered rhGM-CSF subcutaneously for five days were treated with rhGM-CSF in vitro and assessed for migration to 0.1  $\mu$ M FMLP. Figure 38 shows that after a 5 minute exposure to rhGM-CSF in vitro, PMNs from the monkey receiving 50,000 U/kg/day increased only 37.2% over controls in contrast to the 362% increase seen in the saline treated monkey run in parallel. Monkeys receiving 15,000 U/kg/day rhGM-CSF had increases in migration of 25.5% and 151% after 5 minutes exposure to 10 U/ml rhGM-CSF.

c. Effects of In Vivo Administration of rhGM-CSF on Formylpeptide Receptor Expression

Binding studies were performed with PMNs from two monkeys administered rhGM-CSF in vivo. Results from both monkeys indicate a trend towards increased specific binding of  $^3\text{H}$ -FMLP, however these increases varied over the timecourse of the experiment and were often quite small. Interestingly, increases in binding were seen up to 6 weeks after cessation of rhGM-CSF treatment. Figures 39 A and B represent the data from 2 animals. Specific binding for untreated monkeys was 9.1%.

Figure 38. The effects of in vivo administration of various doses of rhGM-CSF for 5 days on priming of rhesus monkeys PMNs in vitro. PMNs were isolated from the peripheral blood of monkeys after receiving subcutaneous b.i.d. injections of rhGM-CSF or saline for 5 days. Purified PMNs were then pretreated with 10 U/ml of rhGM-CSF in vitro for 0, 5, 15, and 60 minutes and assessed for migration to 0.1  $\mu$ m FMLP. Data are expressed as the percent difference between responses of control cells incubated with no rhGM-CSF and those in the presence of 10 U/ml rhGM-CSF at corresponding timepoints. Each value represents the response of an individual animal based on the mean of three replicates.



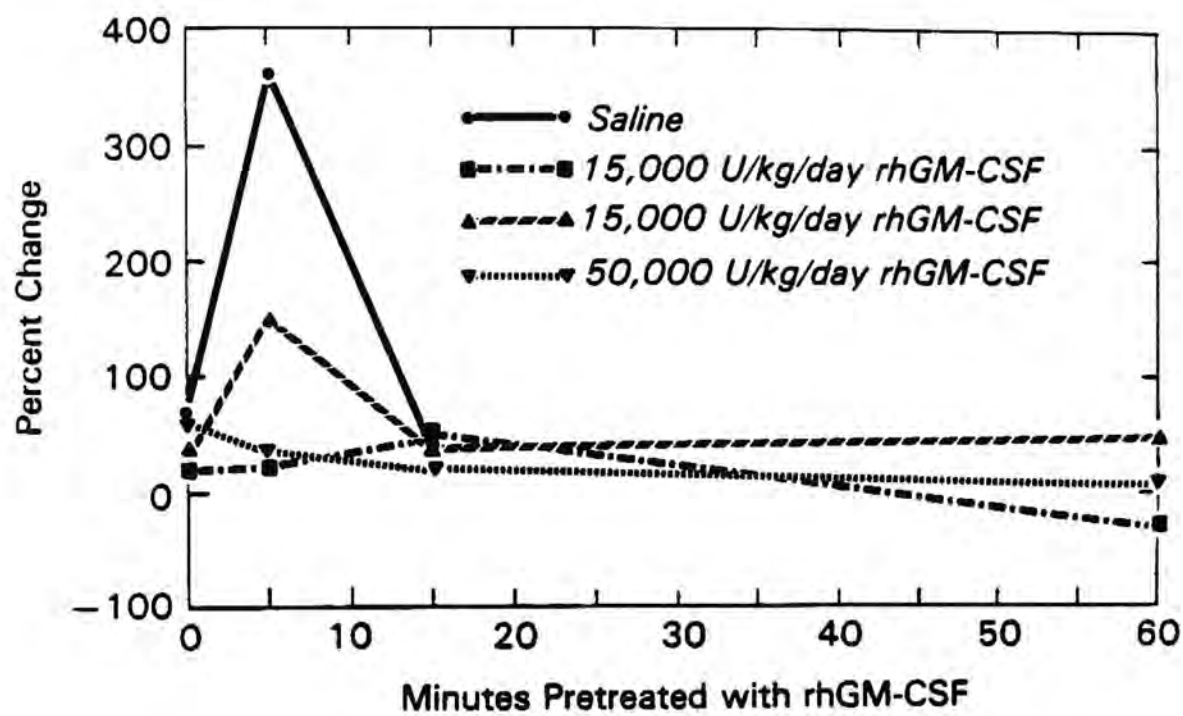
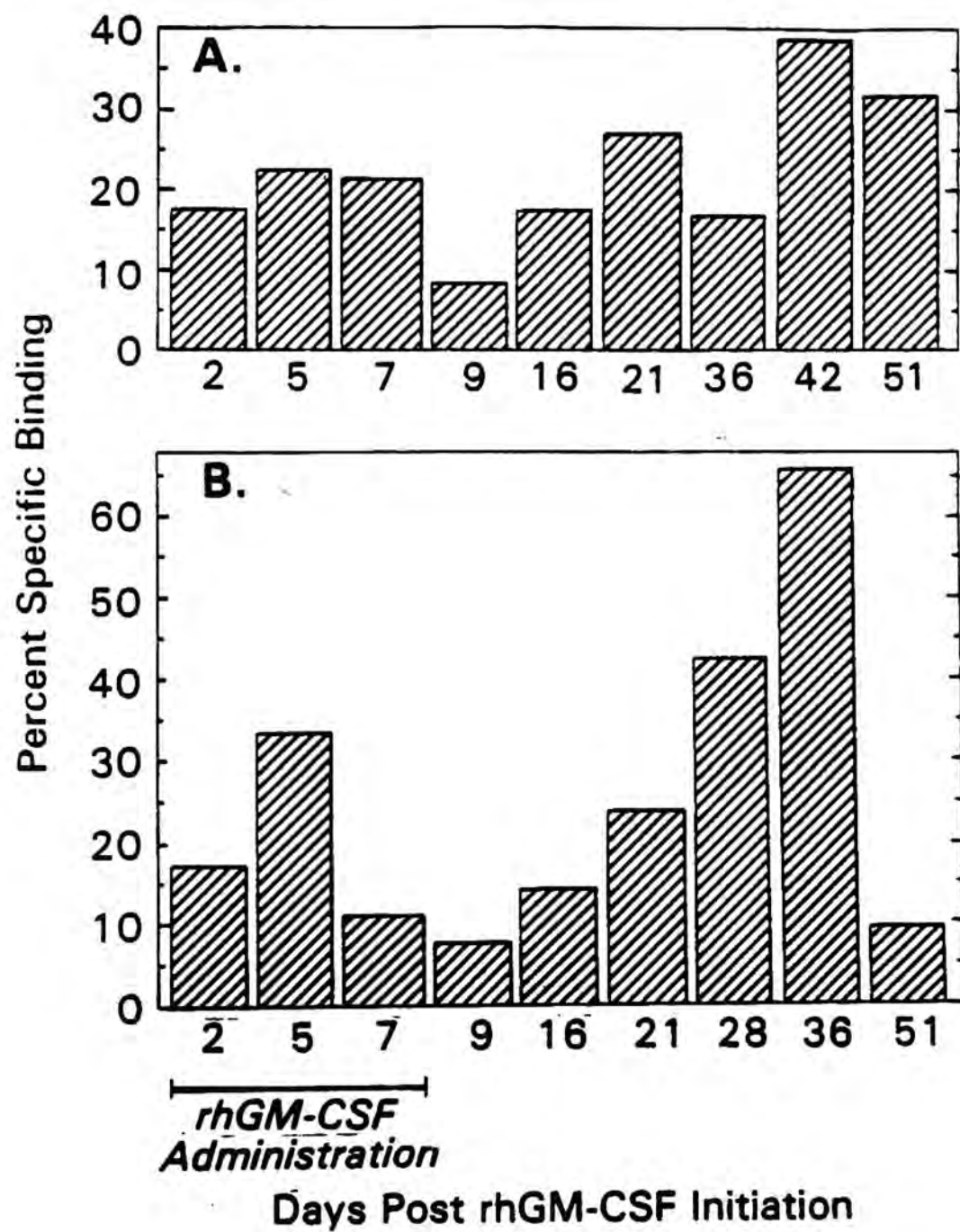


Figure 39. Changes in specific binding of 100 nM  $^3\text{H}$ -FMLP to PMNs obtained from rhesus monkeys after initiation of in vivo rhGM-CSF treatment . Each panel (A and B) reflects data obtained from 1 monkey. RhGM-CSF was administered through day 7. Specific binding of FMLP to untreated monkey PMNs was 9%.



## VII. DISCUSSION

### A. SUMMARY

Comparative studies were conducted assessing functional responses to FMLP of PMNs from peripheral blood of humans, dogs and rhesus monkeys. Motility and production of reactive oxygen intermediates were the parameters examined and the results were compared to formylpeptide receptor expression. Dog PMNs were nonresponsive to concentrations of FMLP shown to stimulate PMNs from humans as well as other species. These observations were supported by an absence of specific binding of  $^3\text{H}$ -FMLP. Rhesus monkey PMNs were poorly responsive to FMLP and had only 9% specific binding of  $^3\text{H}$ -FMLP. This data suggested that the poor responses to FMLP by these species were related to defects in the formylpeptide receptor expression, affinity or signaling.

Pretreatment of dog PMNs with concentrations of PMA as low as 1 pM caused significant increases in migration to FMLP. PMA induces mobilization of human PMN intracellular formylated peptide receptor stores. A23187, an agent which also increases expression of cytoplasmic stores of formylpeptide receptors (5, 81-83), did not affect migration of dog PMNs to FMLP. These data suggest that PMA induced enhancement in migration to FMLP are not mediated through increases in receptor numbers but through the effects of PKC on receptors on the dog

PMN membrane. Subsequent work found that dog PMNs migrated and generated an oxidative burst to high concentrations of FMLP (0.1-1 mM), to high affinity FMLP analogs and to E. coli filtrates, demonstrating the



existence of low affinity or small numbers of formylpeptide receptors on the dog PMN membrane. Figures 40 and 41 are schematics summarizing potential cellular and biochemical mechanisms mediating priming of dog PMN migration to FMLP by PMA.

Recombinant human granulocyte-macrophage colony stimulating factor (rhGM-CSF) has been identified as a priming agent in in vitro studies of human PMN function (88, 89, 138-141). Human rhGM-CSF is also a potent stimulant of hematopoiesis in rhesus monkeys (123, 124), while activity in the dog may be questionable. Studies with the rhesus monkey PMN were undertaken to examine if functional responses of PMNs from a species poorly responsive to FMLP could be enhanced using a molecule known to be involved in inflammatory processes in situ. The effect of in vitro and in vivo treatment with rhGM-CSF on responses to FMLP of PMNs from rhesus monkeys was assessed. In vitro exposure of rhesus monkey PMNs to rhGM-CSF led to significant increases in migration to FMLP after 5 minutes pretreatment while increased oxidative responses were noted only after 60 to 120 minutes pretreatment with rhGM-CSF. RhGM-CSF acted through recruitment of nonresponsive PMNs as well as enhancement of reactive oxygen intermediate production in previously responsive populations. Increases in rhesus monkey PMN binding of  $^3\text{H}$ -FMLP could not be demonstrated after in vitro pretreatment with rhGM-CSF, however formylpeptide receptor expression below the sensitivity of the binding assay is possible. RhGM-CSF increases cGMP levels intracellularly through activation of guanyl cyclase. Enhanced PMN responses have been associated with increases in cGMP. These events are illustrated in Figures 40 and 41 respectively.

Figure 40. This schematic illustrates potential cellular events mediating PMA or rhGM-CSF enhancement of FMLP responsiveness in dog or monkey PMNs. Panel A represents a PMN prior to stimulation. Specific and nonspecific granules are shown as well as expression of formylpeptide receptors on the membrane. It should be noted that there are tremendously reduced levels of formylpeptide binding sites when compared to human PMNs. Cytoplasmic stores of formylpeptide receptors are shown associated with the Golgi. Panel B illustrates increases in FMLP binding sites resulting from mobilization of intracellular receptor stores after stimulation with PMA or rhGM-CSF. Specific granule release results from PMA stimulation but not rhGM-CSF treatment. A second possible mechanism shown in Panel C is an increase in formylpeptide receptor affinity resulting from protein kinase C phosphorylation or events secondary to the activation of PKC. Specific granules are released but there is no expression of cytoplasmic receptor populations.

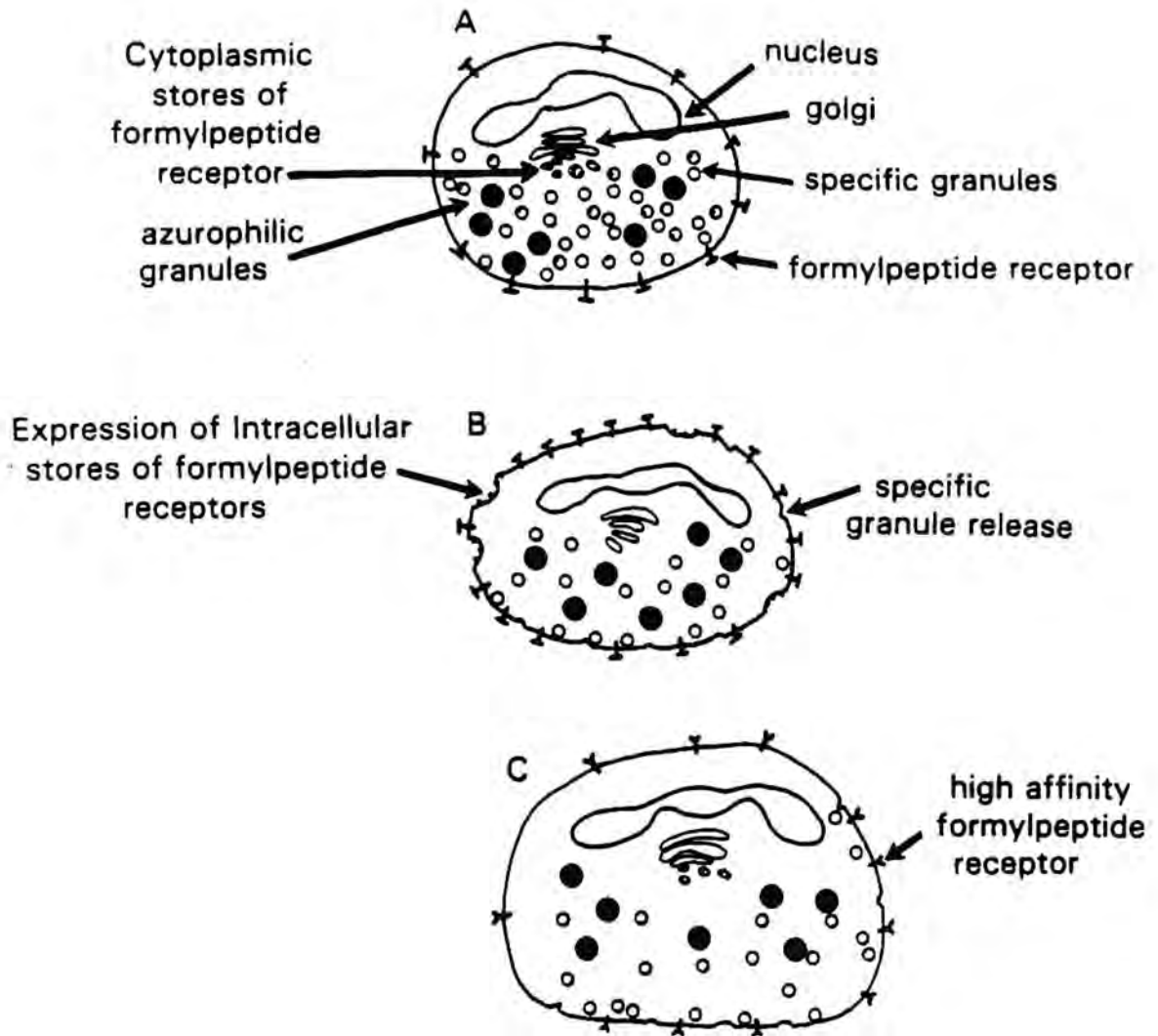
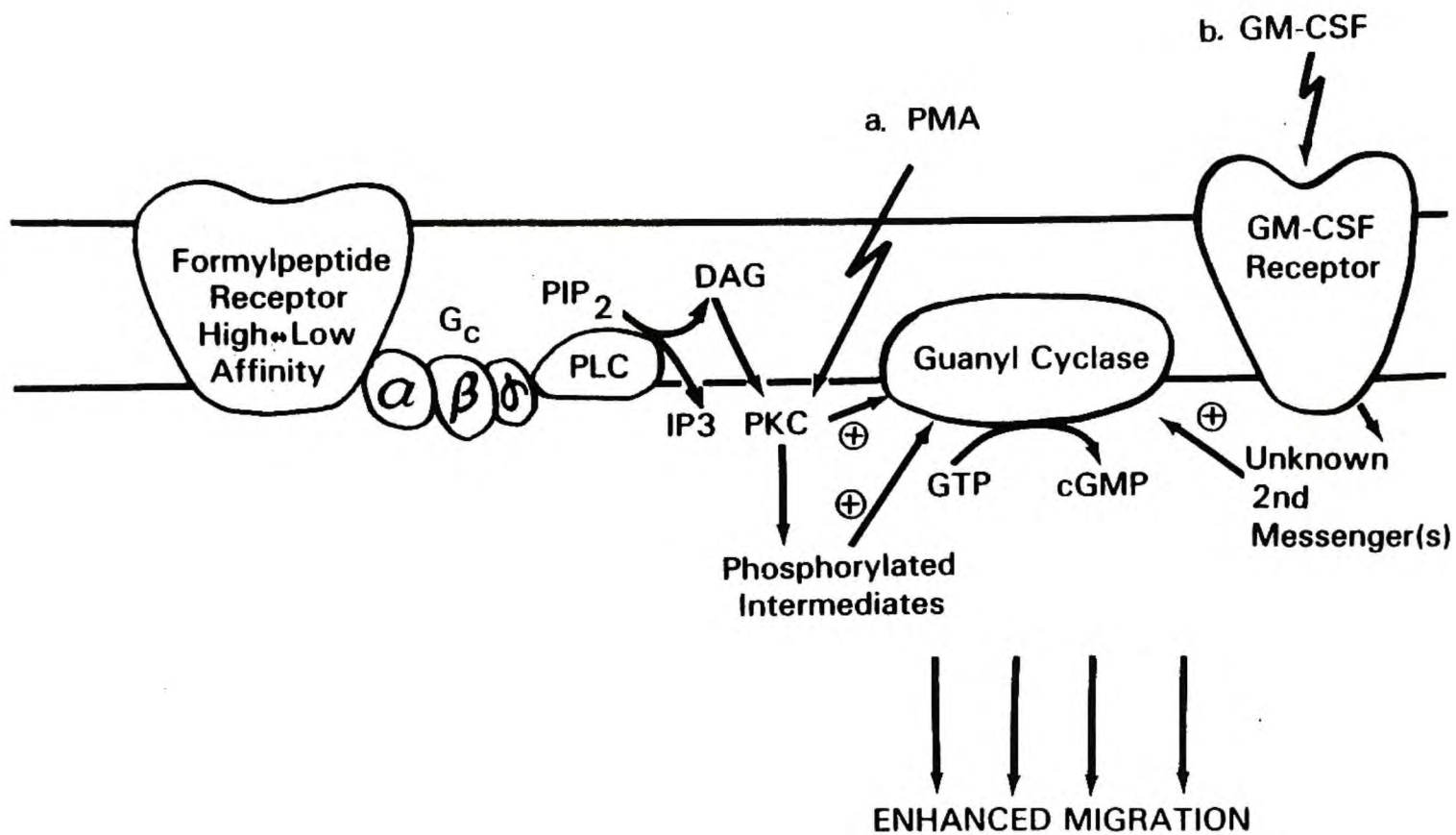


Figure 41. A schematic of potential molecular events involved in PMA (scheme A) or rhGM-CSF (scheme B) mediated amplification of chemotactic receptor signaling. Scheme A shows PMA activating PKC and the subsequent stimulation of guanyl cyclase through direct (PKC) or indirect mechanisms resulting in increases in cGMP and enhanced migration. Scheme B illustrates rhGM-CSF activation of guanyl cyclase through unidentified second messengers generated after the growth factor binds to the GM-CSF receptor.





In vivo administration of rhGM-CSF increased FMLP induced migration and oxidative burst responses after 5 and 7 days. Concomitant increases in random migration at these timepoints suggested the production and release of a more activated population of PMNs.

These studies demonstrate that species poorly responsive to FMLP can be induced to a responsive state after pretreatment with an appropriate priming agent both in vitro and in vivo.

## B. COMPARATIVE STUDIES OF PMN RESPONSES TO FMLP

Studies were conducted comparing the responses to FMLP by PMNs from the peripheral blood of humans, monkeys and dogs. Dog PMNs were nonresponsive to FMLP, rhesus monkey PMNs were poor responders and PMNs from humans responded vigorously (Figures 9-12).

FMLP did not stimulate migration of dog PMNs at concentrations of 1 pM to 1 uM (Figure 9). Migration of dog PMNs to PAF (Figure 1) and to NDS (Figure 2) established that the absence of migration to FMLP was not the result of an intrinsic impairment in motility. Fewer dog PMNs migrated randomly or to stimulants than PMNs from other species (Table 4). Technical studies optimizing the migration assay established that this property was intrinsic to dog PMNs and not a result of the experimental conditions.

The low percentages of dog PMNs migrating both randomly and to stimulants may result from unique features of the dog hematopoietic system. White blood cell counts of dog blood range from 5,000 to 12,000 cells per  $\text{mm}^3$  of which an average of 70% are PMNs (142-144). In addition, blood volume per tissue mass of the dog is high, ranging from 75 to 100 ml/kg

(143). These conditions result in high numbers of PMNs distributed through the tissues when compared to other species. Thus, large numbers of PMNs in relation to body mass, may reduce the necessity for PMN mobile surveillance. Conversely, the cow, a species with low numbers of PMNs in relation to body mass has PMNs with high random mobility (145).

Human PMNs responded vigorously to FMLP. The optimum concentration was between 0.1 and 1  $\mu$ M, values comparable to previous reports (3, 6, 8). The original report of PMN migration to formylated peptides described an  $ED_{50}$  of 70 pM with optimal migration at 10 nM (1). Rabbit peritoneal PMNs, the cell types used for these studies, have since been shown to be more sensitive to FMLP than PMNs from human peripheral blood (8). Rhesus monkey PMNs migrated to FMLP but the response was significantly less than human PMNs (Table 4). Migration of rhesus monkey PMNs was significantly greater than random at 0.1, 1 and 100  $\mu$ M FMLP (Figure 11). Maximum migration was found at 100  $\mu$ M FMLP.

The plateau shaped dose response curve of rhesus monkey PMN migration to FMLP (Figure 11) contrasts sharply with the bell shaped curve dose response observed with human PMNs (Figure 10). The dose response curve for production of the oxidative burst to FMLP by both human and rhesus monkey PMNs is sigmoidal (Figure 12). Physiologically, the relationship between these two responses produces PMNs migrating through a concentration gradient of small amounts of stimulant, becoming immobilized at higher stimulus concentrations, releasing reactive oxygen intermediates, granules containing acid hydrolases and becoming bactericidal.

Interestingly, there were differences in the dose response curves for migration of monkey PMNs to FMLP, ZAS and PAF. As seen in human PMN migration, the dose response curve for rhesus monkey PMN migration to PAF, a phospholipid, was bell shaped (Figure 4). In contrast, the dose response curve of migration to ZAS, a protein (Figure 5), was plateau shaped, as was that to FMLP. These results suggest an absence of rhesus monkey PMN desensitization to high concentrations of protein stimulants. Desensitization of PMN migration at high concentrations of stimulant is key to the maintenance of PMNs at an inflammatory locus. Events mediating the desensitization of motility have yet to be elucidated. Stimulation of migration elicits a number of events at the membrane level. Included among these are changes in expression of membrane components associated with adherence such as C3b and Mo-1 (147). Increases in adherence are associated with migration responses and are necessary for motility since PMNs "crawl" across a substrate as opposed to the "swimming" of bacteria. Too much adherence anchors a PMN to the substrate and inhibits motility. One explanation for the absence of rhesus monkey PMN desensitization to FMLP and ZAS is that these molecules did not cause increases in the expression of adherence proteins at high concentrations of stimulant.

Evidence exists indicating that intracellular signaling for chemotaxis in response to C5a and FMLP is different than  $LTB_4$  (148, 149). A second possible explanation for an absence of rhesus monkey PMN desensitization is that the biochemical events mediating desensitization to the phospholipid PAF may differ from peptides such as FMLP and C5a. Observations such as these highlight the important information which can be obtained through such comparative evaluations. Study of the impaired



desensitization responses by the rhesus monkey PMN could be an interesting means to gain more basic understanding of this process and apply this knowledge to disease states related to dysfunctions of the human PMN.

Few studies exist in the literature describing nonhuman primate PMN function. Gonder et al. (107) reported that maximal migration responses by human and rhesus PMNs were the same. However, higher concentrations of FMLP were required to elicit these responses by rhesus monkey PMNs. The raft technique utilized by Gonder generates a chemotactic index based on the product of cell numbers migrating and distance migrated. The polycarbonate filter assay employed for this project quantitates cell numbers which have migrated and does not account for potential differences in capacity to migrate distances. Suzuki et al. found rhesus monkey PMNs migrating shorter distances to FMLP than human PMNs, also noting migration at concentrations where the human PMNs had desensitized (108).

Greater numbers of rhesus monkey PMNs migrated randomly than human PMNs (Table 4). Studies by Suzuki et al. found that PMNs from four nonhuman primates migrated greater distances in the absence of FMLP (random migration) than human PMNs (108). The inverse relation between random mobility and migration to FMLP may relate to nonspecific immune components existing in a more mobilized state in primates less advanced phylogenetically. Enhanced random migration may deemphasize the importance of directed migration to bacterially derived (i.e., extrinsic) stimulants. Interestingly, migration to PAF and ZAS (Figures 4 and 5) was not enhanced so there is no apparent compensation in PMN response to intrinsic mediators.

Among primates, the ability to migrate to FMLP appears to increase as the species becomes more evolved. This does not apply when considering species outside the primate order, since PMNs from rabbits and guinea pigs are strong responders to FMLP. No common denominator has been identified in the distribution of FMLP responsiveness among species. PMNs obtained from humans (3), chimpanzees (108), rabbits (7) and guinea pigs (9) are quite responsive to FMLP while those from species such as cows (14), dogs (12), sheep (15), cats (16) and pigs (15) are not. The presence of FMLP in bacterial filtrates (150) does suggest a physiological role for this molecule, yet, since no disease states associated specifically with a deficiency in FMLP response have been identified, FMLP responsiveness may not be critical to host defense. Additionally, species nonresponsive to FMLP appear to have sufficient nonspecific immunity. These studies suggest this may result from conversion of FMLP nonresponsive PMNs to a responsive state in the inflammatory milieu. None-the-less, FMLP is an important tool for in vitro investigation of inflammatory cell physiology and species poorly responsive are a unique resource with which to explore stimulus-response coupling in PMNs.

Although migration, degranulation and the production of reactive oxygen intermediates are coupled in the normal human PMN, there are instances where uncoupling of these functions can be demonstrated. Chronic granulomatous disease is characterized by an inability of PMNs to generate a respiratory burst to PMA or receptor mediated stimulants such as FMLP (151), yet PMNs from these patients have the ability to migrate normally (152). Uncoupling of migration from the respiratory burst has also been achieved in vitro, through treatment of normal human PMNs with pharmacological agents, as well as with antibodies against portions of

the formylpeptide receptor (153-157). Naturally occurring models for these types of studies can be found in different species. Studies of the horse PMN have shown normal degranulation to FMLP but an inability to migrate to the chemotactic peptide (11). Therefore, it was important to establish if the species examined in these studies would generate a respiratory burst to FMLP. Control studies with PMA indicated PMNs from dogs and rhesus monkeys were functionally capable of producing a respiratory burst. In contrast, rhesus monkey PMNs were poorly responsive to FMLP and at concentrations of 10 and 100  $\mu$ M of FMLP dog PMNs were nonresponsive (Figure 12). These studies confirmed previous findings indicating that dog PMNs do not migrate or generate an oxidative burst at low concentrations of FMLP (91, 92). The rhesus monkey PMN reduced NBT at concentrations of 10 and 100  $\mu$ M FMLP. Although migration and degranulation of rhesus monkey PMNs to FMLP have been described, only one group has addressed superoxide production by this species (124). These authors made no direct comparisons to human responses, but, the amount of superoxide produced by rhesus monkey PMN was quite low relative to previous reports for human PMNs (124). Figure 12 indicates that the maximum percentages of rhesus monkey PMNs responding to FMLP were less than humans and that the dose response curve was shifted to the right. Pretreatment with cytochalasin B increased the maximum percentage of NBT positive rhesus monkey PMNs and shifted the dose response curve to the left. However, this response was still to the right of the human dose response curve (Figure 6 compared to Figure 12). These results demonstrate that in the absence of cytochalasin B, the maximum percentage of responding rhesus monkey PMNs were less than the human. The results of studies pretreating with cytochalasin B indicate that 100% of



the rhesus monkey PMNs could be induced to respond to FMLP as in the human, although at higher stimulus concentrations. Therefore, the difference in responsiveness to FMLP between rhesus monkey and human PMNs was not due to differences in distribution of PMN subpopulations with the capacity to produce and express formylpeptide receptors.

A comparison of formylpeptide receptor binding data between PMNs from humans, dogs and rhesus monkeys showed that the poor responsiveness of these species correlated with an absence of specific binding of the chemotactic peptide. Dog PMNs did not have detectable specific binding sites for  $^3\text{H}$ -FMLP. Binding of chemotactic peptide to rhesus monkey PMNs had not previously been reported. Receptors for formylpeptide were demonstrated, although there was only 9% specific binding of 100 nM  $^3\text{H}$ -FMLP to rhesus monkey PMNs. The high nonspecific binding prevented analysis of the data with Scatchard plots. Consequently there was no means by which to estimate receptor numbers or affinities. Affinity labeling of the formylpeptide receptor with  $^{125}\text{I}$ -FNLLPNLTL indicated that the rhesus monkey PMN has approximately 25% the amount of formylpeptide receptor as the human PMN (146). Results obtained in binding studies using human PMNs were similar to those found in other laboratories (8). The results of these binding studies suggest either low numbers or low affinity receptors for FMLP on the rhesus monkey PMN.

Ligand elicited signal transduction in the PMN is composed of a sensing, transducing and effector elements. The sensory elements are receptors for specific stimuli such as FMLP, PAF,  $\text{LTB}_4$ , C5a and other mediators. The transducer has been identified as a G protein similar to  $\text{G}_i$  yet coupled to a phosphodiesterase effector element which generates  $\text{IP}_3$  and



DAG as second messengers. Potential mechanisms for decreased functional responses lie at the level of the receptor, G protein or effector elements. These data demonstrate that PMNs from dogs and rhesus monkeys have the capacity to migrate to ligands and generate reactive oxygen intermediates in response to either ligands or PMA. Thus, these cells have functional transduction and effector mechanisms for migration and the oxidative burst. The absence or small amount of specific FMLP binding suggests that the poor functional responses to FMLP observed with the dog and rhesus monkey PMNs are related to the formylpeptide receptor. If receptors exist on the membrane, as some of these studies suggest, then the numbers are below the threshold of the binding assay. Alternately, there may be insufficient amounts of G protein to maintain the receptor in a high affinity configuration. The role of G proteins in mediating the binding affinity of formylpeptides to human PMNs has been demonstrated (4).

These comparative studies largely confirmed previous reports in the literature. Variability in results among laboratories emphasized the importance of performing these experiments with protocols developed for this project as well as properly documenting the baseline control responses for subsequent priming experiments. Comparative studies established that the dog was virtually nonresponsive to FMLP and the rhesus monkey was poorly responsive. Results from human controls were within ranges previously published. This work established that FMLP nonresponsiveness is based on a defect associated with the formylpeptide receptor. Truncated formylpeptide receptor expression, a reduction in affinity of the receptor or insufficient generation of second messengers after receptor binding may be potential causes. The hypothesis of this project proposes that PMA and

rhGM-CSF enhance functional responses of PMNs of species normally refractory to FMLP as a result of changes in formylpeptide receptor numbers, affinity or signal transduction.

### C. ENHANCEMENT OF DOG PMN MIGRATION

Studies were conducted investigating the ability of PMA and the calcium ionophore A23187 to enhance dog PMN migration to FMLP. This work demonstrated that dog PMNs pretreated with PMA increased migration to low concentrations of FMLP. Figures 14 B and C illustrate 1 and 10 nM PMA significantly increased dog PMN migration to FMLP. Maximum increases were seen after pretreatment for 5 minutes with 1 nM PMA in response to 1 pM FMLP where the mean stimulation ratio increased from  $1.2 \pm 0.16$  to  $2.13 \pm 0.33$  (Figure 14B). Responses were dose related as evidenced by the increases found with 1 and 10 nM PMA, while 0.1 nM PMN did not increase migration (Figure 14 A-C). Several groups have reported PMA effective in enhancing migration of human PMNs. Estensen et al. found that human PMN migration to E. coli filtrates in modified Boyden chambers increased dramatically in the presence of 1.6 and 16 nM PMA and after 5 to 10 minute pretreatment with 0.16 and 1.6 nM PMA (158). Work by Gallin et al. illustrated similar effects of a 30 minute pretreatment with 0.16 nM PMA on migration to C5a using a leading edge technique for migration assessment (5). PMA raises cGMP levels in PMNs, as do agents such as acetylcholine, carbamylcholine, imidazole and 8-bromo cyclic GMP (158, 159). Pretreatment of PMNs with these compounds enhances human PMN migration to E. coli filtrates (158, 159). The data presented here

supported the hypothesis that a species nonresponsive to FMLP can be converted to a responsive state.

An absence of specific binding of FMLP to PMNs has led previous investigators to conclude dog PMNs do not have formylpeptide receptors. These studies are the first to examine the possibility that receptor expression could be induced in a nonresponsive species such as the dog. Significant increases in FMLP binding after PMA pretreatment were not observed with dog PMNs. However, there was a trend towards an increased binding (Figure 16). Few numbers of formylpeptide receptors are necessary to elicit migration, therefore motility enhancement could result from increased receptor expression which remains below the limits of detection of the binding assay. The specific activity of  $^3\text{H}$ -FMLP is 55-65 Ci/mM at a concentration of  $2 \times 10^{-5}$  M in ethanol. Labeling of FMLP with isotopes such as  $^{125}\text{I}$  would be necessary to make accurate estimations of small numbers of receptors. The presence of functional formylpeptide receptors in the absence of significant  $^3\text{H}$ -FMLP binding is supported by the migration and production of reactive oxygen intermediates of rhesus monkey PMNs (Figures 11 and 12). These observations support the possibility that increased formylpeptide receptor expression mediates the PMA induced migration enhancement, but are not definitive proof.

A23187, a calcium ionophore, also enhances FMLP responses in human PMNs through mobilization of intracellular receptor stores (81-83). A23187 does not activate PKC as does PMA (160), so the absence of a significant migration enhancement to FMLP by the calcium ionophore (Figure 17 A-C) suggests the responses found with PMA may be related to PKC modification of formylpeptide receptor affinity or events associated with cGMP increases intracellularly.

Pretreatment of human PMNs with n-butanol significantly increased specific binding of FMLP (61, 62). Cryptic receptors in the PMN membrane have been suggested as responsible for these changes. Preliminary studies with the aliphatic alcohol n-butanol did not result in increased binding of FMLP by dog PMNs. These studies indicate that the dog PMN has no such cryptic receptor population in the membrane. Alternately, the membrane constituents of the dog PMN may be different from that of the human and different agents may be necessary to cause the appropriate fluidity changes for expression of cryptic membrane stores.

The enhancement of dog PMN migration to FMLP by PMA suggested that formylpeptide receptors may exist in the dog PMN membrane. Previous comparative work showed an absence of functional responses to FMLP at concentrations less than 1  $\mu$ M, yet two reports in the literature (12, 113) indicated that dog PMNs migrated to E. coli filtrates. Formylated peptides had been identified as an active component of bacteria filtrates in 1975 by Schiffmann and coworkers (1). Studies for this project found that dog PMNs migrated vigorously to E. coli filtrates but comparison of Figures 19 and 20 demonstrate that 50-fold more filtrate was required to elicit maximum dog PMN migration than necessary for human PMNs. Interestingly, the difference in FMLP concentration stimulating maximum PMN migration in dog (1 mM shown in Figure 24) and human (0.1  $\mu$ M shown in Figure 10) was 10,000-fold. These results strongly suggest that a component of the E. coli filtrate was priming the dog PMN response to FMLP in the filtrate. The inhibition of migration to E. coli filtrates by pertussis toxin and the formylpeptide receptor antagonist t-boc-MLP shown in Figures 21 and 23 demonstrate that migration was receptor mediated. Dog PMNs migrated to concentrations of FMLP greater



than 10  $\mu$ M (Figure 24) and in the presence of cytochalasin B, 100  $\mu$ M FMLP stimulated NBT reduction by dog PMNs (Figure 26). Thus, as in humans, formylpeptide receptors on dog PMNs couple to both migration and the oxidative burst. Dog PMNs also migrated to formylpeptides with high affinity for the formylpeptide receptor. The order of potency of these analogs for dog PMNs was the same as has been observed for rabbit PMNs (116, 119), FMLPP > FMLPB > FMLP, (Table 5). FNLLPNLTL has not been assessed for effects on rabbit PMNs, yet, the hexapeptide had a greater potency in humans PMNs than FMLP (74), as was also found for dog PMNs (Table 5). Although the  $ED_{50}$  of the dog PMN was 100- to 1000-fold greater than the human or rabbit PMN, the similarities in response potency of the dog PMN with PMNs from FMLP responsive species further supports formylpeptide receptors on dog PMNs (Figure 27, Table 5). Migration to FMLP, FMLPP and FMLPB was inhibited by pertussis toxin (Figure 29).

Motility is composed of two components, that which is directional (chemotaxis) and that of velocity (chemokinesis) (130, 161, 162). In these studies, human PMN chemotactic migration to FMLP was significantly greater than chemokinetic, however chemokinesis was 75% of the total migration measured. Dog PMN chemotaxis to 0.1 mM FMLP, as well as that to two of the three formylated peptide analogs was significantly greater than chemokinesis. However, chemokinesis was a large percentage of the motility response (Figure 28). The pertussis toxin and antagonist data indicate these responses were receptor mediated and were not nonspecific activation (Figure 28 and 29). One shortcoming of the migration assays using membrane filters is the difficulty in discerning chemotaxis and chemokinesis. An important consideration in utilization of the

polycarbonate assay technique is the small distance (10  $\mu$ M) cells migrate to reach an endpoint. Increases in migration, irrespective of chemotactic or chemokinetic, can both translated into increased numbers of cells migrating. Complications such as these make a definitive statement concerning the directional capacity of the dog PMN migrating to FMLP impossible. It is important to note that increases in both chemotaxis and chemokinesis are advantageous to the host. All PMN stimulants stimulate both chemotaxis and chemokinesis (130). This is of physiological benefit since increases in velocity (chemokinesis) as well as directional migration (chemotaxis) would aid in elimination of potentially pathogenic organisms.

In summary, a 5 minute pretreatment with low concentrations of PMA enhanced motility of dog PMNs to FMLP and shifted the dose response curve dramatically to the left. Upregulation of formylpeptide receptor numbers is suggested by the binding data. It is also possible PKC may have increased the affinity of formylpeptide receptors. In support of this postulate is the observation that A23187, an agent which mobilizes intracellular receptor stores without activation of PKC, did not produce significant enhancement of migration to FMLP. Migration of dog PMNs to formylpeptide analogs and to E. coli filtrates demonstrates that these cells contain small numbers of functional formylpeptide receptors which may be a target for PKC phosphorylation. PMA also produces an increase in PMN cGMP concentrations through activation of guanyl cyclase (159). In human PMNs, cGMP increases are associated with enhanced PMN responsiveness to stimulants. PMA appears to be enhancing dog PMN response to FMLP through PKC phosphorylation and subsequent activation of a substrate such as guanyl cyclase or possibly others. The

schematic shown in Figure 40 depicts these events at the cellular level, while Figure 41 suggests a model for the biochemical events.

#### D. STUDIES WITH RHESUS MONKEY PMNs

The rhesus PMN is poorly responsive to FMLP in both motility (Figure 11) and oxidative burst assays (Figure 12). Studies were undertaken to determine if rhGM-CSF could enhance rhesus monkey PMN responses to FMLP and to further characterize these responses. RhGM-CSF has been shown to activate as well as prime human PMNs in numerous assay systems (88, 89, 121, 138-141). The rhesus monkey has been an important species for use in evaluating the in vivo hematological effects of rhGM-CSF (123).

In vitro pretreatment of monkey PMNs with 100 pM (10 U/ml) rhGM-CSF increased migration to FMLP after 5 minutes preincubation and this effect was time and dose dependent (Figure 30). Figure 31 shows similar effects on migration to PAF were observed after in vitro treatment with rhGM-CSF. RhGM-CSF has been shown to increase expression of the adherence proteins Mo-1, GFA-1 and GFA-2 in a timeframe similar to the migration enhancement reported here (138, 139). The absence of significant changes in random migration after in vitro pretreatment with rhGM-CSF indicates effects on FMLP and PAF elicited migration were ligand specific and not mediated through upregulation of adherence proteins (Figure 31). These results are consistent with observations that human PMN migration to FMLP was enhanced after a 5 to 15 minute pretreatment with 100 pM rhGM-CSF (89). Previous reports have shown that rhGM-CSF decreases the area that PMNs randomly migrate under agarose (140). A rhGM-CSF

mediated decrease in cell numbers migrating randomly was not observed in this study (Figure 31).

PMNs pretreated with rhGM-CSF for varied time intervals were assessed for the ability to generate a respiratory burst in response to FMLP. RhGM-CSF pretreatment resulted in a time related increase in FMLP induced NBT positive cells (Figure 32). The PMN has a key role in inflammation and it is attractive to postulate that GM-CSF amplifies the effects of PMN stimulants in the early stages of an inflammatory response. In vitro studies of stimulated lymphocytes, macrophages and endothelial cells indicate these cells produce GM-CSF (122). In vivo studies have shown that LPS administration elicits a 1000-fold increase in GM-CSF in less than three hours (122). Interleukin-1 and tumor necrosis factor, molecules with potent inflammatory actions, have been shown to stimulate production of GM-CSF by endothelial cells (122). Results from this project demonstrate rapid exposures to rhGM-CSF enhance motility while more prolonged exposures enhance oxidative metabolism (Figure 35). These data suggest that increases in GM-CSF concentration in vivo lead first to enhanced migration to PMN stimulants such as FMLP and PAF, subsequently increasing numbers of PMNs accumulating at an inflammatory site. With a longer exposure to GM-CSF and moving closer to the inflammatory locus, PMNs "switch" from motility to increased production of reactive oxygen intermediates. The appropriate elicitation of motility versus production of reactive oxygen intermediates is critical to host defense. Indiscriminate production of reactive oxygen intermediates in the vasculature, particularly that of the lung, leads to disease states such as Adult Respiratory Distress Syndrome (ARDS).



The terms priming and recruitment have often been used interchangeably, referring to enhancement of PMN responses to a particular stimuli. Numerous investigators have described increased production of superoxide anion by a variety of stimuli after preincubation with LPS,  $LTB_4$ , lipooxygenase products or PAF (91, 100-102). Other groups have reported recruitment of cell populations from a nonresponsive state to that of responsive by  $LTB_4$  and rhGM-CSF (99, 141). Assay systems such as the SOD inhibitable reduction of cytochrome C by superoxide anion yield important information concerning the amount and rate of superoxide production, yet no data are available as to percentages of cells actually responding. The distinction between conversion of nonresponsive to responsive versus enhancement of previous responses is important. Appreciation of the dynamics of heterogeneity in functional responses of PMN populations is a crucial component to understanding the inflammatory process (163). Weisbart et al. (88, 89) demonstrated that 120 minutes of rhGM-CSF pretreatment enhanced superoxide production of human PMNs by FMLP,  $LTB_4$  and C5a while 15 minutes pretreatment increased chemotactic indices of PMNs migrating to FMLP. Although the enhancing effects of rhGM-CSF are very clear, these studies did not permit determination of priming versus recruitment. Figure 33 shows, that in the absence of modulating agents, 32.3% of the monkey PMNs responded to 0.1  $\mu$ M FMLP in contrast to 94% of human PMNs responding under the same conditions (Figure 12). Pretreatment with rhGM-CSF for 120 minutes increased percentages of responsive rhesus monkey PMNs to 87.1% (Figure 33). Clearly, this represents recruitment of PMN populations previously FMLP nonresponsive to a responsive state and stands in strong support of the hypothesis of this project. Recent reports indicated that human PMNs

could be recruited to depolarize in response to FMLP after rhGM-CSF pretreatment yet there was no enhancement of depolarization by those populations previously responding (i.e., priming) (141). Table 6 shows that rhGM-CSF pretreatment resulted in increased percentages of PMNs responding to FMLP (87.1% vs. 30.0%) as well as a darker staining intensity (4.17 vs 1.55). Migration data from this project demonstrates recruitment of PMNs migrating to FMLP and the data of Weisbart (89) showed that cells also migrate greater distances (i.e., priming) after rhGM-CSF pretreatment. These studies indicate that rhGM-CSF both recruited and primed PMN migration and generation of the respiratory burst to FMLP.

An unexpected finding of these studies was the production of small amounts of reactive oxygen intermediates by monkey PMNs after rhGM-CSF pretreatment. Significant increases in numbers of NBT positive PMNs as well as increased DCF fluorescence, indicative of hydrogen peroxide production, occurred after 120 minutes pretreatment with rhGM-CSF (Figures 33 and 36). RhGM-CSF did not stimulate motility of monkey PMNs but has previously been reported to increase expression of adherence proteins (138, 139), increase phagocytosis (139) and increase antibody-dependent cytotoxicity of mature human PMNs (139).

The effects of in vitro treatment of rhesus monkey PMNs on formylpeptide receptor expression are not clear. These studies demonstrated no increases in  $^3\text{H}$ -FMLP binding. However, as previously discussed, the technical limitations of the binding assay may have prevented observing increases. RhGM-CSF has been shown to increase numbers of FMLP binding sites on human PMNs. Therefore, rhGM-CSF mediated increases



in rhesus monkey PMN responses to FMLP may have resulted from enhanced formylpeptide receptor expression as illustrated in Figure 40 B.

The demonstration of a rhGM-CSF mediated increase in cGMP levels by Coffey et al. (164) also suggests increases in cGMP caused migration enhancement through effects distal to the chemotactic receptor (Figure 41). Pretreatment of human PMNs with 100 U/ml rhGM-CSF caused increases in cGMP biphasically over a 1 to 120 minute timecourse (164). Peaks in cGMP accumulation occurred after pretreatment for 10 and 60 minutes (164). These peaks correspond roughly to timepoints where the optimum priming effects for both motility and the oxidative burst have been found (Figure 35). Work performed in the early and midseventies indicates that agents causing increases in cGMP are associated with a variety of enhanced PMN responses (158, 159). Thus, cGMP may prove a common link to the enhanced responses of both dog and rhesus monkey PMNs to FMLP after pretreatment with PMA and rhGM-CSF respectively.

In vivo administration of rhGM-CSF has been shown to stimulate a significant leukocytosis both in normal monkeys (123) and human patients (125). To further address the physiological significance of rhGM-CSF enhancement of PMN responses to FMLP it was important to assess effects of in vivo administration on PMN migration and production of reactive oxygen intermediates. Preliminary clinical studies in human cancer patients showed margination of circulating PMNs 5 minutes after rhGM-CSF injection (165). This response was reversed within an hour. These in vivo studies corresponded well with the rapid changes in PMN function elicited by rhGM-CSF found in the in vitro studies. In vivo administration of rhGM-CSF to normal rhesus monkeys led to significant increases in the ability of isolated PMNs to migrate to both FMLP and

PAF on days 5 and 7 of treatment (Figures 37 A and B). Random migration was also increased on treatment days 5 and 7. Mayer et al. had shown enhanced oxidative metabolism in rhGM-CSF treated rhesus monkeys (124) but effects on motility had not yet been addressed. Binding studies suggested upregulation of formylpeptide receptor expression during rhGM-CSF administration. It should be noted, however, that increased specific binding was quite variable over time as illustrated in Figure 39 A and B.

RhGM-CSF administered both in vitro (Figure 30) and in vivo (Figure 37 A and B) increased rhesus monkey PMN migration to FMLP and PAF. Figure 37 A and B demonstrates in vivo administration of rhGM-CSF required 5 to 7 days before a maximal increase in PMN migration was obtained. This observation, in addition to the concomitant increases in random migration on days 5 and 7, suggests that prolonged exposure to rhGM-CSF may be stimulating the production and release of a more activated population of PMNs. Studies of PMNs obtained from rhesus monkeys administered rhGM-CSF for 5 days indicated these cells had lost the capacity to be further recruited by rhGM-CSF in vitro (Figure 38). Increases in peripheral counts throughout rhGM-CSF treatment have shown that the bone marrow remained responsive to GM-CSF (123) so desensitization appears restricted to mature PMNs.

The PMN has an important role in the first 24 hours of a bacterial challenge and accumulating data suggest GM-CSF helps accelerate this response. GM-CSF is produced by macrophages, endothelial cells and lymphocytes after exposure to compounds such as endotoxin and interleukin-1 (122). In vitro studies suggest enhanced PMN responses to stimulants such as FMLP are rapid effects of GM-CSF and occur through a direct mechanism in the early stages of inflammation. Indeed, GM-CSF receptors



have been identified on human PMNs (166). The in vivo studies show that prolonged elevation of GM-CSF, while a dramatic stimulant to the hematopoietic compartment (123), downregulates the ability of GM-CSF to modulate mature PMN responses to inflammatory mediators. This condition also leads to the production and release of a more aggressive population of PMNs from the bone marrow which are more randomly motile as well as capable of amplified responses to mediators such as FMLP. These studies conclusively prove that rhGM-CSF enhances FMLP responses of the rhesus monkey PMN and suggest a physiological role for GM-CSF in recruiting and priming cells otherwise poorly responsive to FMLP.

In conclusion, these studies establish that the dog and monkey PMN are poorly responsive to FMLP, that the defect is at the level of the formylpeptide receptor and that these nonresponsive species can be converted to a responsive state after pretreatment with either PMA or rhGM-CSF. Although receptor upregulation may have a role in these events, this is not definitively proved by these studies. There is, however, a common link of cGMP increases associated with each treatment which enhances FMLP responses in both the dog and rhesus monkey PMN.

The use of species such as the dog and the rhesus monkey as large animal preclinical models necessitates a stronger data base characterizing PMN function. Additionally, the poor responses of PMNs of these species to FMLP suggest these as interesting models to study defects of PMN function. These studies demonstrate that impaired PMN function can be reversed and suggest receptor expression and cGMP increases as possible mechanisms. Further work more precisely identifying mechanisms mediating enhancement of responses to FMLP by refractory species could

yield important insight into basics of PMN function and provide important information in treatment of human disease states associated with PMN dysfunction.

#### E. PROSPECTUS

The capacity of phagocytes to migrate has been investigated by scientists interested in inflammation for close to 100 years. The discovery a decade ago that motility was initiated by specific membrane bound receptors was a major advance in understanding this critical event of nonspecific immunity.

The objective of these studies was to capitalize on the naturally occurring diversity among species in responsiveness to FMLP, the best characterized of the known PMN stimulants and develop a new approach in the study of stimulus-response coupling of PMN motility. These studies asked if a species poorly or nonresponsive to FMLP could be converted to a responsive state. This work demonstrates first that this can be achieved in vitro and suggests these events may occur and be of significant benefit to the host during inflammation. The evolutionary pressure leading to the species specificity of FMLP responsiveness is unknown. Understanding the factors responsible for this adaptation could yield important insight into the nonspecific immune system. In the absence of such information, research investigating diversity in FMLP responsiveness may generate answers to questions basic to human PMN chemotactic receptor modulation. Data from this project suggests several interesting directions for subsequent research. When tools such as the cDNA for the formylpeptide receptor gene are available, it would be fascinating to test if PMNs from FMLP

nonresponsive species have this gene. These studies, as well as those of others support the hypothesis that some of the FMLP nonresponsive species can express the gene for the formylpeptide receptor. Although PMNs from the peripheral blood of cows do not migrate to FMLP (14), sperm from bulls migrate to FMLP through a receptor with structure activity relations identical to the human PMN formylated peptide receptor (167, 168) suggesting cows also contain the formylpeptide receptor gene. Additionally, pretreatment of calf aortic endothelium with FMLP modifies adherence of PMNs, also suggesting formylpeptide receptors on cow endothelium. Indeed, Rotrosen et al. (146) have characterized formylpeptide receptors on human endothelium. An absence of formylpeptide receptors in PMNs from species which do contain the gene may occur for a number of reasons. Transcription defects, a mRNA splicing defect, instability of the mRNA, instability of the protein or a defect in localization (targeting) of the protein to the cell membrane are each possibilities. Additionally, tissue specific factors required for the synthesis or expression of formylpeptide receptors may be lacking in the PMNs of nonresponsive species.

The regulation of chemotactic receptor synthesis, expression and turnover are virtual black boxes in PMN physiology. Study and understanding of defects in nonresponsive species may provide a foothold into unscrambling and understanding each of these events in the human PMN.



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